

Alpha-2-Macroglobulin Therapies and Drug Screening Methods for Alzheimer's Disease

Background of the Invention

Field of the Invention

5 This invention relates to the field of medical genetics. More specifically, the invention provides for therapeutic agents for Alzheimer's Disease and methods of screening for therapeutic agents for Alzheimer's disease that are based on affecting alpha-2-macroglobulin function and expression.

Related Applications

10 This application is a continuation-in-part of Application Serial No. 09/148,503, filed on September 4, 1998, which claims priority to U.S. Provisional Application No. 60/057,655, filed on September 5, 1997, and U.S. Provisional Application No. 60/093,297, filed on July 17, 1998, all of which are herein incorporated by reference.

Related Art

15 Alzheimer's disease (AD) is a devastating neurodegenerative disorder that affects more than 4 million people per year in the US (Döbeli, H., *Nat. Biotech.* 15: 223-24 (1997)). It is the major form of dementia occurring in mid to late life: approximately 10% of individuals over 65 years of age, and approximately 40%
20 of individuals over 80 years of age, are symptomatic of AD (Price, D. L., and Sisodia, S. S., *Ann. Rev. Neurosci.* 21:479-505 (1998)).

25 The first recognized clinical symptom of AD is usually the loss of short-term memory (Schellenberg, G.D., *Proc. Natl. Acad. Sci. USA* 92:8552-559 (1995)). Other common symptoms include abnormal judgement and behavior, and difficulty with language, orientation, problem solving, calculations, and visuospatial perception (Price, D. L., and Sisodia, S. S., *Ann. Rev. Neurosci.* 21:479-505 (1998); Schellenberg, G.D., *Proc. Natl. Acad. Sci. USA* 92:8552-559 (1995)). These symptoms often worsen until cognitive function is almost entirely lost, and the patient cannot function independently (Schellenberg, G.D., *Proc.*

Natl. Acad. Sci. USA 92:8552-559 (1995); Price, D. L., and Sisodia, S. S., *Ann. Rev. Neurosci.* 21:479-505 (1998)). By late stages of the disease patients typically lack verbal ability, cannot recognize people, and are incontinent and bed-ridden (Price, D. L., and Sisodia, S. S., *Ann. Rev. Neurosci.* 21:479-505 (1998); Sloane, P. D., *Am. Family Phys.* 58: 1577-86 (1998)).

Known risk factors for AD include age, genetic predisposition, abnormal protein (β -amyloid) deposition in the brain, and certain environmental factors such as head injury, hypothyroidism, and a history of depression. The majority of AD patients do not exhibit symptoms until their seventies (Price, D. L., and Sisodia, S. S., *Ann. Rev. Neurosci.* 21:479-505 (1998)). However, individuals who have inherited particular genetic defects often exhibit symptoms in midlife (Price, D. L., and Sisodia, S. S., *Ann. Rev. Neurosci.* 21:479-505 (1998)). This latter type of AD, called early-onset familial AD (FAD), accounts for 5-10% of AD cases, and has been linked to defects in three different genes, *APP*, *PSEN1*, *PSEN2* (Blacker, D. and Tanzi, R. E., *Archives of Neurology* 55:294-296 (1998)). Mutations in these genes lead to increased production of the amyloidogenic β -amyloid peptide ($A\beta$) (Citron, M., *et al.*, *Nature Medicine* 3:67-72 (1997); Suzuki, N., *et al.*, *Science* 264:1336-1340 (1994)).

The most prevalent form of AD, called late-onset AD (LOAD), accounts for approximately 90% of AD cases, and has been genetically linked to *APOE* and *LRP* (Kang, D. E., *et al.*, *Neurology* 49:56-61 (1997); Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995)). Recently, another gene, the alpha-2-macroglobulin gene (*A2M*), was found to be linked to LOAD (Blacker, D., *et al.*, *Nature Genetics* 19:357-360 (1998)). Carriers of a particular mutation in *A2M* were discovered to be at increased risk of AD. This mutation is a pentanucleotide deletion at the 5' splice site of the second exon encoding the bait region of alpha-2-macroglobulin (α_2M), and is referred to as the *A2M-2* genotype. The *A2M-2* genotype is present in 30% of the population (Blacker, D., *et al.*, *Nature Genetics* 19:357-360 (1998)). The *A2M-2* pentanucleotide deletion is a predisposing factor for AD.

Presently, there is no cure for AD on the horizon and its incidence is increasing as the population ages (Price, D. L., and Sisodia, S. S., *Ann. Rev.*

Neurosci. 21:479-505 (1998)). Due to the lateness in life of the onset of AD symptoms, the ability to delay onset by as little as 5 years could decrease the number of AD patients by as much as 50% (Marx, J., *Science* 273:50-53 (1996)). With the large number of people already affected, and projected to be affected by AD, a drug that could merely delay the onset of AD would be very valuable.

Therapeutic agents based on predisposing factors of AD might be able to prevent, delay or slow progression of the disease. However, presently, available treatments are primarily aimed at treatment of the symptoms of the disease (Enz, A., "Classes of drugs," in: *Pharmacotherapy of Alzheimer's Disease*, Gauthier, S., ed., Martin Dunitz, publ., Malden, MA (1998)). These AD drugs offer only modest success, and at most, merely slow the progression of the disease (Delagarza, V. W., *Am. Family Phys.* 58:1175-1182 (1998); Enz, A., "Classes of drugs," in: *Pharmacotherapy of Alzheimer's Disease*, Gauthier, S., ed., Martin Dunitz, publ., Malden, MA (1998)). Presently approved and investigational drugs for treating AD can be characterized as those whose actions enhance neurotransmitter effect, or those believed to protect neurons (Delagarza, V., *Am. Family Phys.* 58:1175-1182 (1998)). The most well known drugs in the first category are the cholinesterase inhibitors, such as tacrine (Cognex™) and donepezil (Aricept™), both of which have been approved by the FDA (Delagarza, V., *Am. Family Phys.* 58:1175-1182 (1998); Sloan, P., *Am. Family Phys.* 58:1577-1586 (1998)). Tacrine and donepezil are only modestly effective (Sloan, P., *Am. Family Phys.* 58:1577-1586 (1998)), and are associated with unpleasant side effects including nausea and vomiting (Delagarza, V., *Am. Family Phys.* 58:1175-1182 (1998)). Several neuro-protective drugs are under investigation for the treatment of AD, including estrogen, vitamin E, selegiline and non-steroidal anti-inflammatory drugs (NSAIDs) (Sloan, P., *Am. Family Phys.* 58:1577-1586 (1998); Delagarza, V., *Am. Family Phys.* 58:1175-1182 (1998)). None of these drugs have been approved yet for the treatment of AD, and each has significant drawbacks, including negative side-effects, or association with increased risk of other diseases. (Sloan, P., *Am. Family Phys.* 58:1577-1586 (1998); Delagarza, V., *Am. Family Phys.* 58:1175-1182 (1998); Enz, A., "Classes

of drugs," in: Pharmacotherapy of Alzheimer's Disease, Gauthier, S., ed., Martin Dunitz, publ., Malden, MA (1998)).

Thus, there is a need for new AD therapeutic agents, especially those based on predisposing factors of AD. In addition, there is a need for drug screening systems to aid in developing these therapeutic agents.

Summary of the Invention

Based on the finding, described herein, that the *A2M-2* deletion leads to the production of altered α_2 M RNA transcripts and proteins, strategies aimed at replacing or supplementing normal α_2 M function and activities, and/or at suppressing defective α_2 M function in the brain may serve as a means for therapeutically preventing, treating, or even reversing AD neuropathogenesis. In addition, these strategies may be useful for treating other pathologies associated with defective α_2 M function. Moreover, methods described herein may be used to screen for these therapeutic agents. Thus, the invention provides for new therapeutic agents for AD, for pharmaceutical compositions containing these therapeutic agents, for methods of using these therapeutic agents, and for methods of screening for these therapeutic agents.

The first aspect of the invention is to provide for a therapeutic agent for Alzheimer's Disease, where the agent can replace or supplement α_2 M function, or can suppress the expression of *A2M-2*. A molecule that can bind to A β and to LRP may be able to promote clearance of A β through LRP mediated endocytosis. Thus, one embodiment of the invention is an anti-LRP-A β molecule having an A β binding domain, and an LRP binding domain. In a preferred embodiment of the invention, this molecule is a peptide.

In one embodiment of the invention the peptide is an anti-LRP-A β peptide having an A β binding domain composed of 10-50 contiguous residues of SEQ ID NO:6, and an LRP binding domain comprising 10-50 contiguous residues of SEQ ID NO:8, which encompass residues 1366-1392 of SEQ ID NO:8. In another embodiment of the invention, the anti-LRP-A β peptide has an A β binding domain with an amino acid sequence selected from the group consisting of SEQ ID NO:6,

SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, and SEQ ID NO:26; and an LRP binding domain composed of the amino acid sequence of SEQ ID NO:10. In yet another embodiment of the invention, the anti-LRP-A β peptide has an A β binding domain with an amino acid sequence selected from the group consisting of SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, and SEQ ID NO:26; and an LRP binding domain composed of 10-50 contiguous residues of SEQ ID NO:8.

The A β binding domain may be connected to the LRP binding domain of the anti-LRP-A β molecule by a covalent bond, linker molecule, or linkerless polyethylene glycol. In a preferred embodiment, the A β and LRP binding domains are connected by a peptide bond. In another preferred embodiment of the invention, the A β and LRP binding domains are connected by a peptide composed of 1-20 glycine residues.

In another embodiment, the anti-LRP-A β peptide has the amino acid sequence of SEQ ID NO:14. Alternatively, the anti-LRP-A β peptide has an A β binding domain having an amino acid sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, and SEQ ID NO:26; an LRP binding domain having the amino acid sequence of SEQ ID NO:10; and a linker connecting the A β binding domain to the LRP binding domain.

In addition, the invention provides for pharmaceutically acceptable salts of the anti-LRP-A β peptide and for nucleic acid molecules encoding the anti-LRP-A β peptide.

Another embodiment of the invention relates to a nucleic acid molecule encoding an anti-LRP- β peptide, where the A β binding domain is encoded by 30-150 contiguous nucleotides of SEQ ID NO:5, and the LRP binding domain is encoded by 30-150 contiguous nucleotides of SEQ ID NO:7. In another embodiment of the invention, the region of the nucleic acid molecule encoding the A β binding domain has a nucleotide sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:17, SEQ ID

NO:19, SEQ ID NO:21, SEQ ID NO:23, and SEQ ID NO:25; and the region encoding the LRP binding domain has the nucleotide sequence of SEQ ID NO:9. In yet another embodiment of the invention, the region of the nucleic acid molecule encoding the A β binding domain has a nucleotide sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, and SEQ ID NO:25; and the region encoding the LRP binding domain is encoded by 30-150 contiguous nucleotides of SEQ ID NO:7. In another embodiment of the invention, the nucleic acid molecule has the nucleotide sequence of SEQ ID NO:13.

The region encoding the A β binding domain may be connected to the region encoding the LRP binding domain of the nucleic acid molecule by a phosphodiester bond. Alternatively, these regions may be connected by a nucleotide encoding a linker peptide. In a preferred embodiment of the invention, the connecting nucleotide encodes 1-20 glycine residues.

In addition, the invention relates to nucleic acid molecules having at least 95% homology to these nucleic acid molecules.

Another embodiment of the invention relates to a nucleic acid molecule that is a first polynucleotide that hybridizes to a second polynucleotide that is complementary to the nucleic acid molecules described above. In another embodiment of the invention, the nucleic acid molecule is a first polynucleotide that hybridizes to a second polynucleotide that is complementary to the nucleotide sequence of SEQ ID NO:13. In yet another embodiment of the invention, the hybridizing conditions for the hybridization of the first and second polynucleotides are as follows: (a) incubate overnight at 42°C in a solution consisting of 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and a 20 μ g/ml denatured, sheared salmon sperm DNA; and (b) wash at 65°C in a solution consisting of 0.1x SSC.

A related embodiment of the invention is a pharmaceutical composition containing an anti-LRP-A β molecule, and one or more pharmaceutically acceptable carriers. In addition, the invention provides for a pharmaceutical composition containing an anti-LRP-A β peptide, or a pharmaceutically acceptable

salt thereof. In a preferred embodiment, the pharmaceutical composition contains an anti-LRP-A β peptide having an amino acid sequence selected from the group consisting of SEQ ID NO:4 or SEQ ID NO:14, or a pharmaceutically acceptable salt thereof, and one or more pharmaceutically acceptable carriers. The invention also relates to a method of combating Alzheimer's Disease in a subject by administering an anti-LRP-A β molecule, or a pharmaceutically acceptable salt thereof. In a preferred embodiment, the anti-LRP-A β molecule is a peptide. In another preferred embodiment, the anti-LRP-A β peptide is a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:4 or SEQ ID NO:14, or a pharmaceutically acceptable salt thereof.

The invention also relates to an *A2M-2* antisense oligonucleotide designed to target *A2M-2* RNA. In one preferred embodiment of the invention, the *A2M-2* antisense oligonucleotide is designed to target *A2M-2* heteronuclear RNA. In another preferred embodiment, the *A2M-2* antisense oligonucleotide is designed to target *A2M-2* mRNA. In one embodiment of the invention, the *A2M-2* antisense oligonucleotide designed to target *A2M* hnRNA has the nucleotide sequence of SEQ ID NO:27. The *A2M-2* antisense oligonucleotide is preferably from 8- 50 nucleotides in length, and more preferably is 15-30 nucleotides in length, and is most preferably 15 nucleotides in length. Thus, in another preferred embodiment of the invention an *A2M-2* antisense oligonucleotide designed to target *A2M-2* hnRNA has the nucleotide sequence of the last 15-30 contiguous nucleotides of SEQ ID NO:27. In another embodiment of the invention the *A2M-2* antisense oligonucleotide designed to target *A2M-2* has the sequence of nucleotides 36-50 of SEQ ID NO:27 or of nucleotides 20 -50 of SEQ ID NO:27. The invention also relates to a pharmaceutical composition containing an *A2M-2* antisense oligonucleotide, and one or more pharmaceutically acceptable carriers. In addition, the invention relates to a method of combating Alzheimer's Disease in a subject by administering the *A2M-2* antisense oligonucleotide.

The invention also provides for a viral vector carrying a transgene encoding α_2 M, or an anti-LRP-A β peptide. In a preferred embodiment of the invention, the viral vector carries a gene encoding α_2 M. In another preferred

embodiment of the invention, the gene encoding α_2 M has the nucleotide sequence of nucleotides 44-4465 of SEQ ID NO:1. The invention also relates to a viral vector carrying a gene encoding an anti-LRP-A β peptide. In another preferred embodiment of the invention, the viral vector is an adeno-associated virus. In addition, the invention provides for a pharmaceutical composition containing the viral vector, and one or more pharmaceutically acceptable carriers, and for a method of combating Alzheimer's Disease in a subject by administering the viral vector.

The second aspect of the invention is to provide for a method of screening for therapeutic agents for Alzheimer's Disease that can replace or supplement α_2 M function, or can suppress the expression of *A2M-2*. One embodiment of the invention is a method of screening for a therapeutic agent for AD by incubating a cell that is heterozygous or homozygous for the *A2M-2* allele in the presence of a test agent, and then determining whether the ratio of normal to aberrant *A2M* mRNA has increased relative to the ratio of normal to aberrant *A2M* mRNA found in cells untreated with the test agent. In one preferred embodiment of this method, the cells are glioma cells. In another preferred embodiment, the cells are hepatoma cells. In yet another preferred embodiment of the invention, the cells are heterozygous for the *A2M-2* allele.

In a related embodiment of this method, S1 nuclease is used to determine the ratio of normal to aberrant *A2M* mRNA, and the probe used is complementary to a nucleotide encoding *A2M* (SEQ ID NO:1). Thus, in one embodiment of the invention, S1 nuclease analysis using a probe complementary to SEQ ID NO:1, where the probe encompasses nucleotides 2057-2284 of SEQ ID NO:1, is used to determine whether the ratio of normal to aberrant *A2M* mRNA has increased. In a preferred method of the invention, the probe used in the S1 nuclease analysis is 300 bp long. In another embodiment of the invention, the probe used in the S1 nuclease analysis is complementary to nucleotides 2024-2323 of SEQ ID NO:1.

Alternatively, RT PCR analysis is used to determine whether the ratio of normal to aberrant *A2M* mRNA has increased. In a preferred method of RT PCR analysis, the primers are designed to amplify a region of *A2M* encompassing exons

17-18. In a more preferred method of RT PCR analysis, the amplified region of $A2M$ encompassing exons 17-18 is 300 bp long. In another embodiment of the invention, the primers used for the RT PCR analysis are designed to amplify nucleotides 2052-2289 of SEQ ID NO:1. Another embodiment of the invention relates to the use of a first primer having a nucleotide sequence complementary to nucleotides 2024-2038 of SEQ ID NO:1, and a second primer having the nucleotide sequence of nucleotides 2309-2323 of SEQ ID NO:1 for the RT PCR analysis.

The invention also provides for a method of screening for a therapeutic agent for Alzheimer's disease by incubating α_2M with a test agent, and then determining whether the treated α_2M has undergone a conformational change, or determining whether the treated α_2M can bind to LRP. In a preferred embodiment of the invention, the α_2M treated with a test agent is tetrameric α_2M . In another preferred embodiment of the invention, an α_2M electrophoretic mobility assay is used to determine whether the treated α_2M has undergone a conformational change. In another embodiment of the invention, an ELISA is used to determine whether the treated α_2M can bind to LRP. In a related embodiment of the invention, the ELISA includes the following steps in sequential order: incubating LRP in a well coated with anti-LRP IgG, incubating the well with treated α_2M , incubating the well with anti- α_2M IgG conjugated to an enzyme, and incubating the well with a substrate for the enzyme. In an alternative embodiment, the ELISA includes the following steps in sequential order: incubating a well coated with LRP with treated α_2M , incubating the well with anti- α_2M IgG conjugated to an enzyme, and incubating the well with the substrate for the enzyme. In another embodiment, the ELISA includes the following steps in sequential order: incubating treated α_2M in a well coated with an anti- α_2M IgG specific for activated α_2M , incubating the well with an anti- α_2M IgG conjugated to an enzyme, and incubating the well with a substrate for the enzyme. In another embodiment of the invention, immunoblotting with anti-LRP IgG and anti- α_2M IgG is used to determine whether the treated α_2M can bind to LRP. In yet another embodiment of the invention, a test for the ability of the treated α_2M to undergo

LRP mediated endocytosis is used to determine whether the treated α_2 M can bind to LRP. In another embodiment of the invention, a test for the ability of the treated α_2 M to undergo LRP mediated degradation is used to determine whether the treated α_2 M can bind to LRP.

Brief Description of the Figures

Figure 1. Figure 1 is an autoradiograph depicting the results of ^{33}P -labeled α_2 M mRNA transcripts from *A2M* from human glioma cell lines that express either wild-type *A2M* ((Blacker, D., *et al.*, *Nat. Genet.* 19:357-360 (1998)) or are heterozygous for the *A2M-2* deletion allele obtained by RT-PCR, and separated on a polyacrylamide gel. *A2M-1/2* lines are indicated as lanes marked "2", *A2M-1/1* lines are indicated as lanes marked "1."

Figure 2. Figure 2 is a schematic representation of four of the altered *A2M* transcripts produced by human glioma cell lines expressing the *A2M-2* allele.

Figure 3. Figure 3 is a photograph of immunoblots of media and extracts from CHO cells transfected with α_2 M truncated after exon 18 that were probed with an anti- α_2 M antibody. The anti- α_2 M antibody detected truncated α_2 M in transfected CHO cells. Panel A: cell lysate; Panel B: media; (-) indicates samples from untransfected cells; (wt) indicates samples from cells transfected with full-length α_2 M construct; (Δ) indicates samples from cells transfected with the α_2 M construct truncated after exon 18; m, d and t indicate monomer, dimer and trimer forms of the truncated protein, respectively. These forms of wild type α_2 M are also visible but not marked.

Figure 4. Figure 4 is a photograph of an immunoblot from cell lysates from wild-type cells (*A2M-1*) (lane labeled 1/1) and cells heterozygous for the *A2M-2* deletion (lanes labeled 1/2) probed with an anti- α_2 M antibody. The lane labeled (+) indicates lysate from CHO cells transfected with full length α_2 M, and probed with an anti- α_2 M antibody. The media (data not shown) from *A2M-1* and *A2M-2* cells contained primarily full-length α_2 M monomers, but in the media from the *A2M-2* cells, small amounts of truncated species could also be observed (data not shown).

Figure 5. Figure 5 depicts the α_2 M conformational change induced by protease (represented by the letter P in a circle) cleavage. Note the exposure of the LRP binding domain (represented by \square) after the conformational change.

Figure 6. Figure 6 depicts one possible amino acid sequence for the anti-LRP-A β polypeptide.

Figure 7. Figure 7 is a schematic of the yeast three-hybrid system for detecting the anti-LRP-A β peptide binding to A β and LRP.

Detailed Description of the Preferred Embodiments

Definitions

In the description that follows, a number of terms used in recombinant DNA technology, molecular and cell biology, and pharmacology are extensively used. To provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Nucleotide: "Nucleotide" refers to a base-sugar-phosphate combination. Nucleotides are monomeric units of a nucleic acid sequence (DNA and RNA). The term nucleotide includes deoxyribonucleoside triphosphates such as dATP, dCTP, dITP, dUTP, dGTP, dTTP, or derivatives thereof. Such derivatives include, for example, [α S]dATP, 7-deaza-dGTP and 7-deaza-dATP. The term nucleotide as used herein also refers to dideoxyribonucleoside triphosphates (ddNTPs) and their derivatives. Illustrated examples of dideoxyribonucleoside triphosphates include, but are not limited to, ddATP, ddCTP, ddGTP, ddITP, and ddTTP. According to the present invention, a "nucleotide" may be unlabeled or detectably labeled by well known techniques. Detectable labels include, for example, radioactive isotopes, fluorescent labels, chemiluminescent labels, bioluminescent labels and enzyme labels.

Polynucleotide: A "polynucleotide" is a linear polymer of nucleotides linked by phosphodiester bonds between the 3' position of one nucleotide and the 5' position of the adjacent nucleotide.

Oligonucleotide: "Oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term "oligonucleotide" also includes oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms in that they may exhibit enhanced cellular uptake, increased stability in the presence of nucleases, and other features which render them more acceptable as therapeutic or diagnostic reagents.

Nucleic acid molecule: By "nucleic acid molecule" is meant a polymeric molecule composed of nucleotides. Nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

Complementary: As used herein, "complementary" refers to the subunit sequence complementarity between two nucleic acids, for example, two DNA molecules. When a nucleotide position in both of the molecules is occupied by nucleotides normally capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position. Thus, two nucleic acids are complementary to each other when a substantial number (at least 60%) of corresponding positions in each of the molecules are occupied by nucleotides which normally base pair with each other (for example, A:T and G:C nucleotide pairs).

Hybridization: The terms "hybridization" and "specifically hybridizes to" refer to the pairing of two complementary single-stranded nucleic acid molecules (RNA and/or DNA) to give a double-stranded molecule. These terms are used to indicate that the nucleotides are sufficiently complementary such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its

target nucleic acid sequence to be specifically hybridizable. An oligonucleotide specifically hybridizes to another when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or, in the case of *in vitro* assays, under conditions in which the assays are conducted.

Primer: As used herein "primer" refers to a single-stranded oligonucleotide that is extended by covalent bonding of nucleotide monomers during amplification or polymerization of a DNA molecule. Minisatellite primers used for the amplification of minisatellite dimer, trimer, tetramer, etc., sequences are well-known in the art.

Template: The term "template" as used herein refers to a double-stranded or single-stranded nucleic acid molecule which is to be amplified, synthesized or sequenced. In the case of a double-stranded DNA molecule, denaturation of its strands to form a first and a second strand is performed before these molecules may be amplified, synthesized or sequenced. A primer, complementary to a portion of a DNA template is hybridized under appropriate conditions and the DNA polymerase of the invention may then synthesize a DNA molecule complementary to the template or a portion thereof. The newly synthesized DNA molecule, according to the invention, may be equal or shorter in length than the original DNA template. Mismatch incorporation or strand slippage during the synthesis or extension of the newly synthesized DNA molecule may result in one or a number of mismatched base pairs. Thus, the synthesized DNA molecule need not be exactly complementary to the DNA template.

Amplification: As used herein "amplification" refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence with the use of a DNA polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a DNA or molecule or primer thereby forming a new DNA molecule complementary to a DNA template. The formed DNA molecule and its

template can be used as templates to synthesize additional DNA molecules. As used herein, one amplification reaction may consist of many rounds of DNA replication. DNA amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 5 to 100 "cycles" of denaturation and synthesis of a DNA molecule.

95%, 96%, 97%, 98% or 99% Homology: By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NO:1 can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711. Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the

percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

Polypeptide: A polypeptide is a polymer composed of amino acid monomers joined by peptide bonds.

Peptide Bond: A peptide bond is a covalent bond between two amino acids in which the alpha-amino group of one amino acid is bonded to the alpha-carboxyl group of the other amino acid.

Isolated nucleic acid molecule or polypeptide: a nucleic acid molecule, DNA or RNA, or a polypeptide, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules or polypeptides according to the present invention further include such molecules produced synthetically.

Linker: By "linker" is intended a molecule that connects the LRP binding domain to the A β binding domain of the anti-LRP-A β molecule. When referring to a linker composed of amino acid residues, linker is used to refer to the amino acid residues connecting the two domains. When referring to a nucleic acid encoding a linker, linker refers to the nucleotide sequence encoding the linking amino acid residues. Where the linker is composed of amino acid residues, it will typically consist of one or more glycine residues, or the nucleotide sequence encoding these residues, however, proline may also be used.

Combating Alzheimer's Disease: The term "combating Alzheimer's Disease" is intended to mean a slowing, delaying, or even reversing the AD process. Thus, for example, the therapeutic agents of the invention may be administered either therapeutically in a patient where symptoms of AD are present, or prophylactically, in a subject at risk of developing AD.

Pharmaceutically acceptable carrier: By pharmaceutically acceptable carrier is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material, or formulation auxiliary of any type.

Performed in sequential order: By "performed in sequential order" is intended that the steps described by this term are performed in the order that the steps are recited, but that other unrecited steps may be performed in between the recited steps.

Test agent: By "test agent" is meant any molecule that is of interest for the treatment or prevention of AD, and is to be tested using the screening methods of the invention.

Ranges: various ranges of numbers are described herein. When a range is used, the range of numbers is meant to be inclusive of the boundary numbers. For example, an oligonucleotide composed of nucleotides 20-50 of SEQ ID NO:27, is meant to include nucleotides 20, and 50 and every nucleotide in between.

Other terms used in the fields of recombinant DNA technology, molecular and cell biology, and pharmacology as used herein will be generally understood by one of ordinary skill in the applicable arts.

Alpha-2-macroglobulin

Alpha-2-Macroglobulin (α_2 M) is a 718 kD glycoprotein found at high concentrations in the serum (Borth, W., *FASEB J.* 6:3345-3353 (1992)). The structure of α_2 M consists of four identical 180 kD monomeric units, of 1451 amino acids each (Sottrup-Jensen, L., *et al.*, *J. Biol. Chem.* 259:8318-8327 (1984)). Disulfide bonds link these monomers into dimers, and noncovalent interactions between dimers lead to formation of the functional homotetramer (Harpel, P. C., *J. Exp. Med.* 138:508-521 (1973); Swenson, R. P. and Howard, J. B., *J. Biol. Chem.* 254:4452-4456 (1979)). In addition to the ability to bind A β , α_2 M binds a variety of polypeptides (proteases, growth factors, and cytokines) and ions (Zn, Cu, Fe)(Borth, W., *FASEB J.* 6:3345-3353 (1992); James, K., *Immunol.*

Today 11:163-166 (1990); Parisi, A. F. and Vallee, B. L., *Biochem.* 9:2421-2426 (1970)).

The best studied function of α_2 M is its pan-protease inhibitory activity (Barret, A. J. and Starkey, P. M., *Biochem. J.* 133:709-724 (1973)). A protease molecule binds the bait region of a α_2 M tetramer, amino acids 666-706, and cleaves any of a number of susceptible peptide bonds in this region ((Harpel, P. C., *J. Exp. Med.* 138:508-521 (1973); Barret, A. J. and Starkey, P. M., *Biochem. J.* 133:709-724 (1973); Sottrup-Jensen, L., *et al.*, *J. Biol. Chem.* 264:15781-15789 (1989)). Protease binding and cleavage triggers a large conformational change in the α_2 M/protease complex, referred to as activation, that ultimately results in entrapment of the protease within the tetramer (Figure 5) (Borth, W., *FASEB J.* 6:3345-3353 (1992)). In each monomer a unique β -Cys- γ -Glu thiol ester bond exists between Cys-949 and Glu-952 (Borth, W., *FASEB J.* 6:3345-3353 (1992)). Upon activation this thiol ester bond emerges from a hydrophobic environment and can undergo nucleophilic attack, for example, by lysine residues from the reacting proteases. The result of this nucleophilic attack is a covalent bond between Glu-952 of α_2 M and surface lysine residues of the protease (Figure 5). The protease is effectively trapped, unable to dissociate from α_2 M but still able to cleave small peptide substrates (Qui, W. Q., *et al.*, *J. Biol. Chem.* 271:8443-8451 (1996)). Protease-mediated activation results in exposure of the α_2 M receptor/low density lipoprotein receptor-related protein binding domain (Figure 5) (Strickland, D., *et al.*, *J. Biol. Chem.* 265:17401-17404 (1990)). Low density lipoprotein receptor-related protein (LRP) is a 600 kD endocytic membrane-bound receptor belonging to the low-density lipoprotein receptor family (Borth, W., *FASEB J.* 6:3345-3353 (1992)). LRP is a multifunctional receptor, because it binds ligands from different classes (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995)). Exposure of this LRP binding domain is a prerequisite for LRP mediated endocytosis of α_2 M/ligand complexes and targeted degradation (Borth, W., *FASEB J.* 6:3345-3353 (1992)). In summary, α_2 M serves to bind a number of protein substrates, including A β , and target them for internalization and degradation.

α_2 M binds A β specifically and tightly. The A β binding region of α_2 M is located between residues 1202-1312, approximately 600 residues C-terminal to the bait region (Hughes, S. R., *et al.*, *Proc. Natl. Acad. Sci. USA* 95:3275-3280 (1998)). Binding does not require α_2 M activation and binding stoichiometry is approximately 1.1 A β / mol of α_2 M (Du, Y., *et al.*, *J. Neurochem.* 69:299-305 (1997)). The apparent dissociation constant (K_D) for the A β / α_2 M complex has been reported as 3.8×10^{-10} M for α_2 M/ 125 I-A β (Du, Y., *et al.*, *J. Neurochem.* 69:299-305 (1997)) and 3.5×10^{-7} M for biotinA β /(ruthenium (II) tris-bipyridine-n-hydroxysuccinimide ester) modified- α_2 M (Hughes, S. R., *et al.*, *Proc. Natl. Acad. Sci. USA* 95:3275-3280 (1998)). Despite this discrepancy in K_D values (which are most likely due to methodological differences), a strong interaction between A β and α_2 M exists. This interaction prevents A β fibril formation and fibril associated neurotoxicity ((Hughes, S. R., *et al.*, *Proc. Natl. Acad. Sci. USA* 95:3275-3280 (1998); Du, Y., *et al.*, *J. Neurochem.* 70:1182-1188 (1998)). Recently, it has been demonstrated that a region of α_2 M encompassing only the A β and LRP binding domains is sufficient for A β binding *in vivo* ((Hughes, S. R., *et al.*, *Proc. Natl. Acad. Sci. USA* 95:3275-3280 (1998)). These data suggest that the A β binding domain is an independent structural unit and successful α_2 M/A β interaction may only rely on a few key interactions. Recent work by Soto and colleagues show that an eleven residue peptide is capable of binding A β and inhibiting A β fibril formation (Soto, C., *et al.*, *Nature Medicine* 4:822-826 (1998)), supporting the idea that only a few key interactions are needed to bind A β . In summary, α_2 M can mediate the catabolism of A β in a LRP dependent process.

A2M-2 Genotype

The A2M-2 genotype, which is linked to late-onset AD, is present in 30% of the population (Blacker, D., *et al.*, *Nature Genetics* 19:357-360 (1998)). This genotype has a pentanucleotide deletion at the 5' splice site of the second exon encoding the bait region of α_2 M (exon 18) (Blacker, D., *et al.*, *Nature Genetics* 19:357-360 (1998)).

Low resolution X-ray data and biochemical data suggest that the bait regions are located at the dimer interface and are crucial for the formation of functional tetramers, and the mediation of the conformational change that accompanies activation (Andersen, G. R., *et al.*, *J. Biol. Chem.* 270:25133-25141 (1995); Bowen, M. E. and Gettins, P. G. W., *J. Biol. Chem.* 273:1825-1831 (1998)). The A2M-2 deletion in the bait region could prevent A β clearance and degradation if (i) proteases can not cleave the altered bait region, (ii) protease-induced activation cannot occur, (iii) LRP binding is disrupted, and/or (iv) A β binding is disrupted.

Low density Lipoprotein Receptor-Related Protein

LRP is a 600 kD endocytic membrane-bound receptor belonging to the low-density lipoprotein receptor family (Borth, W., *FASEB J.* 6:3345-3353 (1992)). LRP is expressed in a variety of cell types including: adipocytes, astrocytes, fibroblasts, hepatocytes, macrophages, monocytes, and syncytiotrophoblasts. LRP is translated as a 4525 residue single chain precursor (Nielsen, K. L., *et al.*, *J. Biol. Chem.* 271:12909-12912 (1996)). It is then processed into a 515 kD A chain and an 85 kD β chain. The β chain possesses a single transmembrane segment and a cytoplasmic tail containing two copies of the NPXY endocytosis signal sequence (Nielsen, K. L., *et al.*, *J. Biol. Chem.* 271:12909-12912 (1996)). The extracellularly located α chain contains four cysteine-rich LDL receptor ligand-binding repeats flanked by epidermal growth factor (EGF) repeats (Nielsen, K. L., *et al.*, *J. Biol. Chem.* 271:12909-12912 (1996)). The noncovalent association of the α chain with the extracellular portion of the β chain forms a functional LRP (Borth, W., *FASEB J.* 6:3345-3353 (1992)). LRP is a multifunction receptor because it binds ligands from different classes (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995)). These include α_2 M-protease complexes, plasminogen activator inhibitor-plasminogen activator complexes, lipoprotein lipase, apoE, bovine pancreatic trypsin inhibitor, lactoferrin, Pseudomonas, exotoxin A, nexin-1 complexes, and receptor associated protein (RAP) (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995)). Most of these

ligands do not compete for the same binding site. RAP, however, inhibits the binding of all these ligands.

α_2 M/LRP Association

The association of activated α_2 M and LRP is highly pH dependent, acidification to pH 6.8 or below abolishes binding (Borth, W., *FASEB J.* 6:3345-3353 (1992)). This suggests that upon endocytosis α_2 M dissociates from LRP. After endocytosis α_2 M and its associated ligands are degraded in the lysosome and LRP is recycled to the membrane (Borth, W., *FASEB J.* 6:3345-3353 (1992)). The half-life for internalization and degradation varies between 15 and 60 minutes (Borth, W., *FASEB J.* 6:3345-3353 (1992)).

The α_2 M-protease binding site of LRP has been mapped to residues 776-1399 of the β chain (Nielsen, K. L., *et al.*, *J. Biol. Chem.* 271:12909-12912 (1996)). This region includes EGF repeats 4-6 and LDL receptor ligand binding repeats 3-10. The LRP binding domain of α_2 M is located between residues 1312 and 1451, directly C-terminal to the A β binding domain (Hughes, S. R., *et al.*, *Proc. Natl. Acad. Sci. USA* 95:3275-3280 (1998)). This domain is very flexible relative to the core of α_2 M (Andersen, G. R., *et al.*, *J. Biol. Chem.* 270:25133-25141 (1995)). Low resolution crystal structures (10Å) indicate that activated α_2 M is roughly the shape of an H and the LRP binding domains are located at the tips of the H (Figure 5) (Andersen, G. R., *et al.*, *J. Biol. Chem.* 270:25133-25141 (1995)). A LRP consensus binding sequence has been proposed based on 31 LRP ligands from 7 different protein families (Nielsen, K. L., *et al.*, *J. Biol. Chem.* 271:12909-12912 (1996)). This 27 residue consensus sequence is located between residues 1365 and 1393 of human α_2 M. Once again, experimental evidence suggests that a few key interactions may be important in LRP/ α_2 M binding. Mutations at positions 5 and 10 of the consensus sequence, corresponding to Lys-1370 and Lys-1374 in the human α_2 M, abolish binding unlike mutations at other highly conserved residues.

Implication of α_2M in Alzheimer's Disease

Cerebral deposition of amyloid is a central event in AD (Soto, C., *et al.*, *Nat. Med.* 4:822-826 (1998)). Genetic, neuropathological, and biochemical evidence indicate that inappropriate deposition of amyloid plays a fundamental role in the pathogenesis of AD. The major component of AD amyloid plaques is A β , a 39-43 amino acid peptide. A β polymerizes as dense (amyloid plaque) and diffuse extracellular deposits in the neuropil (Masters, C. L., *et al.*, *Proc. Natl. Acad. Sci. USA* 82:4245-4249 (1985)), and in cerebral blood vessels (congoophilic angiopathy) (Glenner, G. G. and Wong, C. W., *Biochem. Biophys. Res. Comm.* 120:885-890 (1984)) of both AD and Down syndrome (DS) patients. Soluble A β is found in the cerebrospinal fluid (CSF) and is produced (Haass, C., *et al.*, *Nature* 359:322-325 (1992); Seubert, P., *et al.*, *Nature* 359:325-327 (1992); Shoji, M., *et al.*, *Science* 258:126-129 (1992)) by constitutive cleavage of its transmembrane parent molecule, the amyloid protein precursor (APP) (Kang, J., *et al.*, *Nature* 325:733-736 (1987); Goldbarger, D., *et al.*, *Science* 235:877-880 (1987); Robakis, N. K., *et al.*, *Proc. Natl. Acad. Sci. USA* 84:4190-4194 (1987); Tanzi, R. E., *et al.*, *Science* 235:880-884 (1987)). APP is a family of alternatively-spliced proteins, of unknown function, that are ubiquitously expressed (Tanzi, R. E., *et al.*, *Nature* 331:528-530 (1988)). Unknown proteases cleave APP to produce a mixture of A β peptides with carboxyl-terminal heterogeneity. A β 1-40, the major soluble A β species, is found in the CSF at low nanomolar concentrations (Vigo-Pelfrey, C., *et al.*, *J. Neurochem.* 61:1965-1968 (1993)). A β 1-42 is a minor soluble A β species, but is heavily enriched in amyloid plaques (Masters, C. L., *et al.*, *Proc. Natl. Acad. Sci. USA* 82:4245-4249 (1985); Kang, J., *et al.*, *Nature* 325:733-736 (1987); Roher, A. E., *et al.*, *J. Biol. Chem.* 268:3072-3083 (1993)).

The mechanism by which these amyloid deposits result in dementia is unclear, but may be related to the neurotoxic effects of A β at micromolar concentrations (Pike, C. J., *et al.*, *Brain Res.* 563:311-314 (1991)). Insight into the mechanism of amyloid deposit formation began with the discovery of pathogenic mutations of APP close to, or within, the AB domain (van

Broeckhoven, C., *et al.*, *Science* 248:1120-1122 (1990); Levy, E., *et al.*, *Science* 248:1124-1126 (1990); Goate, A., *et al.*, *Nature* 349:704-706 (1991); Murrell, J., *et al.*, *Science* 254:97-99 (1991); Mullan, M., *et al.*, *Nat. Genet.* 1:345-347 (1992)). These studies indicated that the metabolism of A β , and APP, is intimately involved with the pathophysiology of AD. Increasing evidence suggests that increased levels of A β 1-42 accelerates amyloid deposition in early-onset familial AD (FAD). The FAD-linked *APP*670/671 mutation has been shown to increase the secretion of A β species several-fold (Citron, M., *et al.*, *Nature* 360:672-674 (1992)). While the *APP*717 mutation does not affect the quantity of A β production (Cai, X-D., *et al.*, *Science* 259:514-516 (1993)), this mutation increases the proportion of A β 1-42 produced (Suzuki, N., *et al.*, *Science* 264:1336-1340 (1994)). Increased soluble A β 1-42 has also been found in the brains of individuals affected by Down syndrome, a condition complicated by premature AD (Teller, J. K., *et al.*, *Nat. Med.* 2:93-95 (1996)). Inheritance of the other FAD-linked mutations of Presenilin-1 (*PSEN1*) or Presenilin-2 (*PSEN2*) (Sherrington, R., *et al.*, *Nature* 375:754-760 (1995); Levy-Lahad, E., *et al.*, *Science* 269:973-977 (1995)) correlates with increased cortical amyloid burden. The emerging consensus is that the common effect of FAD-linked presenilin mutations is to increase A β 1-42 production (Citron, M., *et al.*, *Nat. Med.* 3:67-72 (1997); Xia, W., *et al.*, *J. Biol. Chem.* 272:7977-7982 (1997)). Taken together these studies suggest that mutations in the genes linked to FAD (*APP*, *PSEN1*, *PSEN2*) can result in increased A β 1-42 production and that this increase could cause FAD. In the vast majority of AD patients, however, overproduction does not occur (Van Gool, W. A., *et al.*, *Ann. Neurol.* 37:277-279 (1995)).

Ninety percent of AD patients suffer from late-onset AD (LOAD). Three genes have been linked to this form of AD: *APOE*, *LRP*, and *A2M*. Inheritance of the *APOE*- $\epsilon 4$ allele on chromosome 19 correlates with increased cortical amyloid burden (Rebeck, G. W., *et al.*, *Neuron* 11:575-580 (1993)). *APOE* promoter polymorphisms, which upregulate transcription of *APOE*, have recently been shown to be associated with AD (Bullido, M. J., *et al.*, *Nat. Genet.* 18:69-71 (1998); Lambert, J. C., *et al.*, *Human Mol. Gen.* 6:533-540 (1998)). Higher

expression of the *APOE-ε4* allele, relative to *APOE-ε3*, has been found in brains of *APOE-ε4* positive AD patients, but not in age- and genotype-matched controls (Lambert, J. C., *et al.*, *Human Mol. Gen.* 6:2151-2154 (1997)). The absence of apoE in transgenic mice expressing FAD mutant APP attenuates Aβ deposition (Bales, K. R., *et al.*, *Nature Genetics* 17:264 (1997)). The second gene linked to LOAD, the *LRP* gene, encodes the low density lipoprotein receptor-related protein. APP, apoE, and α₂M are all ligands for this cell-surface receptor (Blacker, D. and Tanzi, R. E., *Archives of Neurology* 55:294-296 (1998); Kang, D. E., *et al.*, *Neurology* 49:56-61 (1997); Blacker, D., *et al.*, *Neurology* 48:139-147 (1997); Farrer, L. A., *et al.*, *JAMA* 278:1349-1356 (1997); Strittmatter, W. J., *et al.*, *Proc. Natl. Acad. Sci. USA* 90:1977-1981 (1993)). LRP internalizes ligands via endocytosis, and targets them for lysosomal degradation (Borth, W., *FASEB J.* 6:3345-3353 (1992)). Inheritance of a pentanucleotide deletion in the third gene associated with LOAD, *A2M* (*i.e.*, inheritance of *A2M-2*), confers increased risk for AD and is present in ~30% of the population (Blacker, D., *et al.*, *Nat. Genet.* 19:357-360 (1998)). The protein product of *A2M*, α₂M, is an abundant pan-protease inhibitor found primarily in serum, but is also present in brain and other organs (for example, liver). α₂M binds Aβ and can mediate its internalization and degradation (Borth, W., *FASEB J.* 6:3345-3353 (1992); Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911 (1997)).

α₂M has been implicated in the pathogenesis of AD by both biological and genetic findings. α₂M-like immunoreactivity was observed in AD cortical senile plaques (Bauer, J., *et al.*, *FEBS Lett.* 285:111-114 (1991)) and it was shown that α₂M is upregulated in the AD brain where it localizes to neuritic but not diffuse amyloid plaques (Strauss, S., *et al.*, *Lab. Invest* 66:223-230 (1992); Van Gool, D., *et al.*, *Neurobiol. Aging* 14:233-237 (1993)). In addition, Aβ was found to bind to α₂M with high affinity (Du, Y., *et al.*, *J. Neurochem.* 69:299-305 (1997)), and binding prevented amyloid fibril formation as well as neurotoxicity associated with aggregated Aβ (Du, Y., *et al.*, *J. Neurochem.* 70:1182-1188 (1998); Hughes, S. R., *et al.*, *Proc. Natl. Acad. Sci. USA* 95:3275-3280 (1998)). Activated α₂M-Aβ complexes were recently shown to be internalized and targeted for degradation by

glioblastoma cells via binding to LRP (Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911 (1997)). Moreover, LRP is especially abundant in brain regions affected by AD such as the hippocampus (Rebeck, G.W., *et al.*, *Neuron* 11:575-580 (1993); Tooyama, I., *et al.*, *Mol. Chem. Neuropathol.* 18:153-160 (1993)), and serves as a receptor for ApoE (Rebeck, G.W., *et al.*, *Neuron* 11:575-580 (1993)), a well established genetic risk factor (Blacker, D., *et al.*, *Nature Gen.* 19:357-360 (1998)).

The genetic linkage of *APP*, *APOE*, *A2M*, and their receptor *LRP* to AD suggests that these proteins may participate in a common neuropathogenic pathway leading to AD (Blacker, D., *et al.*, *Nat. Genet.* 19:357-360 (1998)). This pathway may be the α_2 M mediated clearance and degradation of A β through α_2 M binding to LRP for endocytosis and lysosomal degradation, and by serving as a direct mediator for A β degradation when α_2 M is complexed with an unidentified serine protease (Qiu, W. Q., *et al.*, *J. Biol. Chem.* 271:8443-8451 (1996)). This hypothesis is supported, *inter alia*, by the fact that apoE and α_2 M are both ligands for *LRP* and, in addition, that apoE has previously been reported to inhibit α_2 M mediated degradation of A β (Rebeck, G. W., *et al.*, *Ann. Neurol.* 37:211-217 (1995); Zhang, Z., *et al.*, *Int. J. Exp. Clin. Invest.* 3:156-161 (1996)).

However, in its normal role, α_2 M also binds a host of cytokines, growth factors, and biologically active peptides (Borth, W., *FASEB J.* 6:3345-3353 (1992)). It has also recently been shown to activate the phosphatidylinositol 3-kinase suggesting a role in signaling (Misra, U. K. and Pizzo, S. V., *J. Biol. Chem.* 273:13399-13402 (1998)). Thus, defective activity of α_2 M may lead to AD-related neurodegeneration by a variety of mechanisms beyond possible effects on A β accumulation and deposition.

A reduced steady-state level of secreted α_2 M or the presence of defective tetramers due to dominant negative effects of *A2M-2* could result in impaired α_2 M function. Partial or total deletion of the sequences coding for the bait region in exons 17 and 18 are likely to modify protease binding, activation, and internalization of potentially defective tetramers containing mutant monomer(s). Therefore, the generation of very low levels of mutant monomers may have an

amplified effect as one mutant monomer may potentially inhibit the function of three wild-type monomers in the tetramer (dominant negative effect). Thus a critical role for α_2 M is indicated in AD neuropathogenesis. The data described in Example 1 show that the *A2M-2* deletion leads to deleted/truncated forms of α_2 M RNA and protein that may have a dominant negative effect on normal α_2 M. Based on the finding, described herein, that the *A2M-2* deletion leads to the production of altered α_2 M transcripts and proteins, strategies aimed at replacing or supplementing normal α_2 M function and activities, and/or at suppressing defective α_2 M function in the brain may effectively serve as a means for therapeutically preventing, treating, or even reversing AD neuropathogenesis. In addition, these strategies may be useful for treating other pathologies associated with defective α_2 M function. Moreover, methods based on the results and experiments described herein may be used to screen for these therapeutic agents.

The first aspect of present invention relates to therapeutic agents for AD that can replace or supplement normal α_2 M function, and/or suppress expression of *A2M-2*.

In one embodiment of the invention, the therapeutic agent is an anti-LRP-A β molecule, which is a molecule containing LRP and A β binding domains. This molecule may be a peptide, or other molecule, that is capable of binding to both A β and LRP. This anti-LRP-A β molecule may also contain other domains. An anti-LRP-A β molecule having A β and LRP binding domains could bind A β and target it for LRP mediated endocytosis followed by lysosomal degradation, and thus would be useful, *inter alia*, as a therapeutic agent.

In one embodiment of the invention, the anti-LRP-A β molecule is a peptide, referred to herein as the anti-LRP-A β peptide. A 250-residue fragment of the α_2 M monomer contains both the A β and LRP binding domains (Hughes, S. R., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 95:3275-3280 (1998)). Thus, in one embodiment of the invention, the anti-LRP-A β peptide would be composed of the entire A β and LRP binding domains of α_2 M (SEQ ID NO:4). Alternatively, the A β and LRP binding domains may be composed of portions of the A β and LRP binding domains of α_2 M. The A β binding domain of α_2 M is located between

residues 1201 and 1313, approximately 600 residues C-terminal to the bait region (Hughes, S.R., *et al.*, *Proc. Natl. Acad. Sci. USA* 95:3275-3280 (1998)). Thus, in another embodiment of the invention, the A β binding domain of the anti-LRP-A β peptide would consist of the full A β binding domain of α_2 M (between residues 1201-1313, SEQ ID NO:6), but only a portion of the LRP binding domain. In another embodiment of the invention, the A β binding domain would consist of at least 50 contiguous residues of the full A β binding domain of α_2 M. In another embodiment of the invention, the A β binding domain would consist of 10-50 contiguous residues of the full A β binding domain of α_2 M.

In addition, peptides that can bind A β in vivo and inhibit A β fibril formation have been described by Soto *et al.* (Soto, C. *et al.*, *Nat. Med.* 4:822-826 (1998); Soto, C., *et al.*, *Biochem. Biophys. Res. Comm.* 226:672-680 (1996)). These peptides (SEQ ID NOs:12, 16, 18, 20, 22, 24 and 26) have homology to A β and a similar degree of hydrophobicity, but have a low propensity to adopt a β -sheet conformation. In particular one 11 residue A β binding peptide, having the amino acid sequence of SEQ ID NO:12, and encoded by the nucleic acid sequence of SEQ ID NO:11, was particularly effective. Therefore, in a preferred embodiment of the invention, the A β domain of the anti-LRP-A β peptide would have the sequence of this 11-residue peptide. Thus, in a preferred embodiment of the invention, the A β domain of the anti-LRP-A β peptide has the amino acid sequence of SEQ ID NO:12, and is encoded by the nucleic acid sequence of SEQ ID NO:11. Two shorter derivatives of this 11 residue A β binding peptide, composed of a 5 residue peptide (SEQ ID NO:22) and a 7 residue peptide (SEQ ID NO:18) also effectively bound A β and inhibited fibril formation (Soto, C. *et al.*, *Nat. Med.* 4:822-826 (1998); Soto, C., *et al.*, *Biochem. Biophys. Res. Comm.* 226:672-680 (1996)). Thus, in another preferred embodiment of the invention, the A β binding domain has the amino acid sequence of SEQ ID NO:22, and is encoded by the nucleic acid sequence of SEQ ID NO:21, or has the amino acid sequence of SEQ ID NO:18, and is encoded by the nucleic acid sequence of SEQ ID NO:17. Alternatively, the A β binding domain may be composed of other derivatives of the 11 residue A β binding peptide having 3, 4 or 6 residues (SEQ

ID NO:24, 22 and 18 respectively). Thus in another embodiment of the invention, the A β binding domain has the amino acid sequence of SEQ ID NO:24, 22 or 18, and is encoded by the nucleic acid sequence of SEQ ID NO:23, 21 or 17, respectively.

5 The LRP binding domain of α_2 M is located between residues 1312 and 1451 of α_2 M, directly C-terminal to the A β binding domain (Hughes, S. R., *et al.*, *Proc. Natl. Acad. Sci. USA* 95:3275-3280 (1998)). Thus, in one embodiment of the invention, the LRP binding domain of the anti-LRP-A β peptide is composed of the full LRP binding domain of α_2 M (residues 1313-1451, SEQ ID NO:8). In
10 another embodiment of the invention, the LRP binding domain is composed of at least at least 50 contiguous residues of the full LRP binding domain of α_2 M. In yet another embodiment of the invention, the LRP binding domain is composed of 10-50 contiguous residues of the full LRP binding domain of α_2 M. Within the LRP binding domain, a 27 residue LRP binding consensus sequence exists at
15 residues 1366-1392 (Nielsen, K. L., *et al.*, *J. Biol. Chem.* 271:12909-12912 (1996)). Thus, in a preferred embodiment of the invention, the LRP binding domain of the anti-LRP-A β peptide is composed of residues 1366-1392 (SEQ ID NO:10) of α_2 M. Alternatively, the LRP binding domain may be composed of a contiguous portion of residues 1313-1451 of α_2 M that includes residues 1366-
20 1392. In another preferred embodiment, the anti-LRP-A β peptide is composed of the 11 residue A β binding domain and the 27 residue consensus sequence of the α_2 M LRP binding domain (SEQ ID NO:14).

 The A β binding domain and the LRP binding domain of the anti-LRP-A β molecule may be connected to each other directly by a covalent bond, or indirectly
25 by another molecule, such as a linker, or linkerless polyethylene glycol. Linker molecules include polymers such as polyethylene glycol (PEG) and peptides or amino acid residues. In addition, linkerless PEG modification (PEGylation) may be used (Francis, G. E., *et al.*, *Int. J. Hematol.* 68:1-18 (1998)). Various methods of connecting molecules using linkers and other molecules are well known in the
30 art, and may be used to connect the A β and LRP binding domains (See, for example, Francis, G. E., *et al.*, *Int. J. Hematol.* 68:1-18 (1998); Raag, R. and

Whitlow, M., *FASEB J.* 9:73-80 (1995); Deguchi, Y., *et al.*, *Bioconjug. Chem.* 10:32-37 (1999); Luo, D., *et al.*, *J. Biotechnol.* 65:225-228 (1998); Reiter, Y., and Pastan, I., *Clin Cancer Res.* 2:245-52 (1996); DeNardo, G. L., *et al.*, *Clin. Canc. Res.* 4:2483-90 (1998); Taremi, S. S., *Protein Sci.* 7:2143-2149 (1998); Schaffer, D. V., and Lauffenburger, D. A., *J. Biol. Chem.* 273:28004-28009 (1998); Skordalakes, E., *et al.*, *Biochem.* 37:14420-14427 (1998); Czerwinski, G., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 95:11520-11525 (1998); Daffix, I., *et al.*, *J. Pept. Res.* 52:1-14 (1998); Liu, S. J., *et al.*, *Blood* 92:2103-2112 (1998); Chandler, L. A., *et al.*, *Int. J. Cancer* 78:106-111 (1998); Park, C. J., *Appl. Microbiol. Biotechnol.* 50:71-76 (1998); Suzuki, Y., *et al.*, *J. Biomed. Mater. Res.* 42:112-116 (1998); Filikov, A. V., and James, T. L., *J. Comput. Aided Mol. Des.* 12:229-240 (1998); MacKenzie, R., and To, R., *J. Immunol. Methods* 220:39-49 (1998)).

In one preferred embodiment of the invention, the linker is composed of amino acid residues, for example, glycine residues or proline residues. Where the linker is composed of amino acid residues, it may be from 1-20 residues, but will preferably be 5-10 residues, and more preferably will be 5 residues.

Where the anti-LRP-A β molecule is a peptide, within the peptide, the A β binding domain may be C-terminal, or N-terminal to the LRP binding domain. However, preferably, the A β binding domain will be N-terminal to the LRP binding domain, which is the order of the A β and LRP binding domains in naturally occurring α_2 M.

In addition, the invention provides for nucleic acid molecules that encode an anti-LRP-A β peptide. Thus, in another embodiment of the invention, the nucleic acid molecules would encode an anti-LRP-A β peptide having the sequences described above. The invention also relates to nucleic acids having at least 95% homology to these nucleic acids. In addition, the invention relates to nucleic acids that hybridize to a nucleic acid that is complementary to a nucleic acid encoding the anti-LRP-A β peptide. The conditions under which the first and second polynucleotides hybridize are preferably as follows: (a) incubate overnight at 42°C in a solution consisting of 50% formamide, 5x SSC, 50 mM sodium

phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and a 20 μ g/ml denatured, sheared salmon sperm DNA; and (b) wash at 65°C in a solution consisting of 0.1x SSC.

The anti-LRP-A β peptide may be produced using standard solid phase synthesis methods for protein synthesis, and purified by high performance liquid chromatography (HPLC) which are well known in the art (See "Preparation and Handling of Peptides," in: *Current Protocols in Protein Science*, Coligan, J. E., et al., eds., John Wiley and Sons, Inc., pub., Vol. 2., Chapter 18 (Suppl. 14 1998)). Alternatively, the anti-LRP-A β peptide may be produced using standard recombinant DNA methods. For example, The DNA coding for the desired sequence of the LRP binding domain (for example, the 27 residue consensus sequence) may be obtained by PCR amplification of the codons encoding the desired LRP binding domain using primers designed to flank the desired codons. This DNA may then be used as a template for PCR mediated integration of the sequence coding for the desired A β binding domain. For PCR mediated insertion of the A β domain, a nucleotide 5' PCR primer can be designed having (1) a region homologous to the end of the DNA sequence encoding the desired LRP binding domain that was amplified as described immediately above, and (2) immediately 5' to this region, a region encoding the desired A β binding domain, and (3) immediately 5' to this region a start codon. For the 3' primer, the 3' flanking primer used to amplify the LRP binding domain, which sequence is now being used as the template, may be used. Alternatively, to produce an anti-LRP-A β peptide having the entire A β and LRP binding domains of α_2 M (residues 1202-1451), primers may be designed to flank the coding sequence for these domains, to amplify this region (nucleotides 3713-4465). A start codon may be then added by PCR mediated insertion. To amplify a coding region that encodes less than the entire AB and LRP binding domains, the primers may instead be designed to flank this smaller region of α_2 M. The resulting nucleic acid molecule is DNA encoding a fusion protein having LRP and A β binding domains, and a start codon, such that this molecule may be inserted into an expression vector to produce the anti-LRP-A β peptide.

Once DNA encoding the desired fusion protein is obtained, PCR mediated insertion may be used to insert restriction enzyme sites at the 5' and 3' ends of the fusion gene so that the fusion protein gene may then be cleaved with these restriction enzymes for insertion into an expression vector, and a vector for use in the yeast three hybrid system (Tirode, F., *et al.*, *J.Biol. Chem.* 272:22995-22999 (1997)). For example, an Xho I and Kpn I restriction sites can be inserted at the 5' and 3' ends of the fusion protein gene, respectively. Cleavage with these restriction enzymes will then facilitate cloning of the fusion protein gene into (i) the pBAD/His expression vector (Invitrogen), for arabinose dependent expression of anti-LRP-A β in *E. coli*, and (ii) the pLex9-3H vector for use in the yeast three hybrid system (Tirode, F., *et al.*, *J.Biol. Chem.* 272:22995-22999 (1997)). The protein product, named anti-LRP-A β peptide, of the resulting gene should have both A β and LRP binding properties.

The ability of anti-LRP-A β molecule to bind A β and LRP and to undergo LRP mediated endocytosis and degradation may be tested using gel-filtration chromatography, immunoblotting and cell culture techniques. If the anti-LRP-A β molecule is a peptide, a yeast-three-hybrid system may also be used to evaluate the anti-LRP-A β peptide (Tirode, F., *et al.*, *J. Biol. Chem.* 272:22995-22999 (1997)). If necessary, the binding properties of an anti-LRP-A β peptide may be reoptimized using *in vivo* evolution techniques (Buchholz, F., *et al.*, *Nat. Biotechnol.* 16:657-662 (1998)).

Gel-filtration chromatography can be performed as described by Narita *et al.* (Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911 (1997)) to test the ability of an anti-LRP-A β molecule to bind A β . The anti-LRP-A β molecule is incubated with A β 1-42 that is radiolabeled with ^3H , ^{14}C or ^{125}I . In the following discussion, ^{125}I -A β is used as an example of radiolabeled A β . Methylamine or trypsin activated $\alpha_2\text{M}$, and $\alpha_2\text{M}$, and unactivated $\alpha_2\text{M}$ and $\alpha_2\text{M-2}$, may be used as controls. anti-LRP-A β / ^{125}I -A β , $\alpha_2\text{M}/^{125}\text{I}$ -A β and $\alpha_2\text{M-2}/^{125}\text{I}$ -A β complexes are then separated from unbound ^{125}I -A β using a Superose 6 gel-filtration column (0.7 x 20 cm) under the control of an FPLC (Pharmacia) that has been standardized with molecular weight markers from 1000 kD- 4 kD. If anti-LRP-

A β has bound ^{125}I -A β , ^{125}I -A β should be detected by gamma counter at two peaks, one corresponding to the molecular weight of the anti-LRP-A β / ^{125}I -A β complex (about 8-9 kD for a complex containing an anti-LRP-A β of about 40 residues), and one corresponding to the molecular weight of ^{125}I -A β (4.5 kD).

Alternatively, or in addition to gel-filtration chromatography, immunoblotting methods (Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911 (1997)) may be used to determine whether an anti-LRP-A β molecule can bind A β . Unlabeled A β is incubated separately with anti-LRP-A β , unactivated $\alpha_2\text{M}$, unactivated $\alpha_2\text{M}$ -2, $\alpha_2\text{M}$ activated by methylamine or trypsin, or $\alpha_2\text{M}$ -2 activated by methylamine or trypsin. Samples are then electrophoresed on a 5% SDS-PAGE, under non-reducing conditions, transferred to polyvinyl difluoride nitrocellulose membrane, and probed with anti-A β IgG, or an antibody specific for the anti-LRP-A β molecule. Where one or more domains of the anti-LRP-A β molecule are derived from $\alpha_2\text{M}$, an anti- $\alpha_2\text{M}$ IgG that recognizes the domain derived from $\alpha_2\text{M}$ may be used, such as anti- $\alpha_2\text{M}$ IgG raised against the LRP binding domain of $\alpha_2\text{M}$ (for example, Marynen, P., *et al.*, *J. Immunol.* 127:1782-1787 (1981)). If the anti-LRP-A β /A β sample may be detected by both the antibody against anti-LRP-A β , and anti-A β IgG it can be concluded that the anti-LRP-A β molecule can bind A β . Where the A β binding domain of the anti-LRP-A β molecule is derived from A β , the anti-A β antibody should be tested to ensure that it does not recognize the anti-LRP-A β molecule. Several antibodies against A β are available, including 6310, WO2, 4G8, G210 and G211. Antibody 4G8 may recognize an anti-LRP-A β molecule for which A β binding domain is derived from A β . In addition, some anti- $\alpha_2\text{M}$ antibodies may not recognize an anti-LRP-A β molecule derived from $\alpha_2\text{M}$, therefore, they should be tested for the ability to recognize the peptide prior to performing the immunoblotting, endocytosis, and degradation protocols described herein. Marynen *et al.*, (Marynen, P., *et al.*, *J. Immunol.* 127:1782-1787 (1981)) describe an anti- $\alpha_2\text{M}$ antibody raised against the LRP binding domain that may be able to recognize an anti-LRP-A β peptide having an LRP binding domain derived from $\alpha_2\text{M}$. Other anti- $\alpha_2\text{M}$ antibodies are available from Sigma and Cortex Biochem (San Leandro, CA, U.S.A.). $\alpha_2\text{M}$ can

be obtained from Sigma, or purified from human plasma and activated as described in Warshawsky, I., *et al.*, *J. Clin. Invest.* 92:937-944 (1993). Synthetic A β ₁₋₄₂ can be purchased from Bachem (Torrance, CA, U.S.A.).

Gel-filtration chromatography and immunoblotting as described above may also be used to determine the ability of anti-LRP-A β to bind LRP, by using labeled soluble LRP (for example, the extracellular region of LRP) in place of labeled A β for gel-filtration chromatography experiments, and anti-LRP IgG in place of anti-A β IgG for immunoblotting experiments. Alternatively, for the immunoblotting protocol, the anti-LRP-A β molecule may be labeled with fluorescent or radioactive label. For a labeled anti-LRP-A β molecule, it can be concluded that the anti-LRP-A β molecule can bind A β if the labeled band corresponds to a band recognized by anti-A β antibody.

The ability of A β /anti-LRP-A β complexes to undergo LRP mediated endocytosis and subsequent degradation can be determined using cell culture experiments using cells that express LRP as described by Kounnas *et al.* (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995); Kounnas, M. Z., *et al.*, *J. Biol. Chem.* 270:9307-9312 (1995)). The amount of radioligand that is internalized or degraded by cells has been described previously (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995); Kounnas, M. Z., *et al.*, *J. Biol. Chem.* 270:9307-9312 (1995)). Cells that express LRP include, but are not limited to, adipocytes, astrocytes, fibroblasts, hepatocytes, macrophages, monocytes, and syncytiotrophoblasts. In one preferred embodiment of the invention, mouse embryo fibroblasts are used for the cell culture experiment.

Cells expressing LRP are incubated for 18 hours with ¹²⁵I-A β (alternatively, A β may be labeled with ³H or ¹⁴C) in the presence or absence of anti-LRP-A β , unactivated α_2 M, unactivated α_2 M-2, α_2 M activated by methylamine or trypsin, or α_2 M-2 activated by methylamine or trypsin; in the presence or absence of RAP (400 nM). RAP is an inhibitor of LRP ligand binding, and is added to determine if endocytosis is LRP mediated. RAP can be isolated and purified from a glutathione *S*-transferase fusion protein expressed in *E. coli* as described in Warshawsky, I., *et al.*, *J. Clin. Invest.* 92:937-944 (1993b). To assess

endocytosis rather than degradation, chloroquine (0.1 mM) is added at the same time as anti-LRP-A β /¹²⁵I-A β to inhibit lysosomal degradation of ¹²⁵I-A β .

The amount of radioactive ligand released by treatment with trypsin-EDTA, proteinase K solution defines the surface-bound material, and the amount of radioactivity associated with the cell pellet defines the amount of internalized ligand. Activated α_2 M/¹²⁵I-A β will serve as positive control. Under the conditions described, more than 8 fmoles / 10⁴ cells of activated α_2 M/¹²⁵I-A β should be internalized after 18 hours of incubation (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995)). Unactivated α_2 M/¹²⁵I-A β will serve as the negative control for endocytosis, because α_2 M must be activated by trypsin or methylamine to be recognized by LRP. If the amount of anti-LRP-A β /¹²⁵I-A β is greater than 4-8 fmoles/10⁴ cells, it can be concluded that anti-LRP-A β /¹²⁵I-A β has the ability to undergo LRP mediated endocytosis. Unactivated α_2 M/¹²⁵I-A β , and activated α_2 M/¹²⁵I-A β in the presence of RAP should not be internalized, therefore no more than 2-4 fmoles/10⁴ cells should be internalized (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995)). Internalization of the anti-LRP-A β /¹²⁵I-A β complex will be deemed abolished if anti-LRP-A β /¹²⁵I-A β , in the presence and absence of RAP, and unactivated α_2 M/¹²⁵I-A β show the same amount of radioactivity associated with the cell pellet.

To determine the ability of A β /anti-LRP-A β complexes to undergo degradation after endocytosis, this cell culture protocol is repeated without chloroquine. The radioactivity appearing in the cell culture medium that is soluble in 10% trichloroacetic acid is taken to represent degraded ¹²⁵I-A β (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995); Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911 (1997)). Total ligand degradation is corrected for the amount of degradation that occurs in control wells lacking cells. Because free ¹²⁵I-A β can be degraded in an LRP independent manner, degradation is measured for anti-LRP-A β and α_2 M complexes with ¹²⁵I-A β , as well as for free ¹²⁵I-A β , in the presence and absence of RAP. Using the same positive and negative controls as above, if RAP does not decrease the amount of TCA soluble radioactivity by at least 30% for the anti-

LRP-A β /¹²⁵I-A β complex, it can be concluded that ¹²⁵I-A β ligand of anti-LRP-A β is not degraded.

Another method of testing the ability of anti-LRP-A β molecule to bind A β and LRP is the yeast three-hybrid system described by Tirode *et al.* (Tirode, F., *et al.*, *J. Biol. Chem.* 272:22995-22999 (1997)). This method may be used where the anti-LRP-A β molecule is a peptide. In this system, yeast growth only occurs when the "bait" recognizes both the "hook" and the "fish" (Figure 7). In this instance, the "hook" is constructed of the DNA coding for A β (Bales, K. R., *et al.*, *Nat. Genet.* 17:264 (1997)), fused to the coding sequence of the LexA DNA binding protein in pLex9-3H, a *TRP1* episomal vector (Tirode, F., *et al.*, *J. Biol. Chem.* 272:22995-22999 (1997)). The "fish" is constructed of the coding sequence for the 515kD extracellular domain of LRP, fused to the B42 activation domain in pVP 16, a *LEU2* episomal vector (Tirode, F., *et al.*, *J. Biol. Chem.* 272:22995-22999 (1997)). The "bait" is the DNA coding for anti-LRP-A β in the pLex9-3H vector, expression of anti-LRP-A β is repressed by methionine. After transformation of yeast with these vectors, transcription of the Leu 2 reporter gene will occur only when the A β fused DNA binding domain is brought into proximity to the transcriptional activation domain fused to LRP. The A β /LRP binding fusion peptide should promote reporter gene transcription. The interaction between anti-LRP-A β and A β and LRP (515 kD) will be considered positive only if reporter gene expression (yeast growth) occurs when A β -LexA, LRP(515kD)-B42, and anti-LRP-A β are expressed.

The anti-LRP-A β molecule of the invention may be administered per se, or in the form of a pharmaceutically acceptable salt with any non-toxic, organic or inorganic acid. Illustrative inorganic acids which form suitable salts include hydrochloric, hydrobromic, sulfuric and phosphoric acid, and acid metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Illustrative organic acids which form suitable salts include the mono, di and tricarboxylic acids. Illustrative of such acids are, for example, acetic, glycolic, lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric, citric, ascorbic, maleic, hydroxymaleic, benzoic, hydroxybenzoic, phenylacetic, cinnamic, salicylic,

2-phenoxybenzoic and sulfonic acids such as methane sulfonic acid and 2-hydroxyethane sulfonic acid. Salts of the carboxy terminal amino acid moiety include the non-toxic carboxylic acid salts formed with any suitable inorganic or organic bases. Illustratively, these salts include those of alkali metals, as for example, sodium and potassium; alkaline earth metals, such as calcium and magnesium; light metals of Group IIIA including aluminum; and organic primary, secondary and tertiary amines, as for example, trialkylamines, including triethylamine, procaine, dibenzylamine, 1-ethenamine, N,N'-dibenzylethylenediamine, dihydroabietylamine, N-(lower)alkylpiperidine, and any other suitable amine.

The amount of the anti-LRP-A β molecule administered to a subject will vary depending upon the age, weight, and condition of the subject. The course of treatment may last from several days to several months or until a cure is effected or a diminution of disease state is achieved, or alternatively may continue for a period of years, for example, when used prophylactically. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. However, the amount of anti-LRP-A β molecule administered to a subject is generally from 0.1 nanograms to 10 mg/kg/day, and is typically an amount ranging from 1 nanogram to 1 mg/kg/day.

The present invention also relates to antisense oligonucleotides targeted to *A2M-2* RNA, and to their use as therapeutic agents for AD and for suppressing *A2M-2* expression. Partial or total deletion of the sequences coding for the bait region in exons 17 and 18 of α_2 M is likely to modify protease binding, interfering with α_2 M activation. Incorporation of one or more mutant monomers into tetramers might thereby result in defective tetramers that could not be activated efficiently and, therefore, could not undergo subsequent endocytosis via LRP. Thus, the generation of very low levels of mutant monomers may have an amplified effect as one mutant monomer may potentially inhibit the function of three wild-type monomers in the tetramer (dominant negative effect). One way to counter this dominant negative effect is to decrease the level of abnormal α_2 M

by interfering with gene expression at the RNA level. For this purpose, an antisense oligonucleotide specific for *A2M-2* RNA can be used. This oligonucleotide will be referred to herein as *A2M-2* antisense oligonucleotide. The *A2M-2* antisense oligonucleotide may be targeted to any *A2M-2* RNA molecule, but in a preferred embodiment of the invention, it is targeted to heterologous nuclear (hnRNA).

The *A2M-2* deletion is found in the splicing region of exon 18, therefore, in one embodiment of the invention, the *A2M-2* antisense oligonucleotide is designed to target *A2M-2* RNA transcripts before splicing occurs, referred to as hnRNA. In addition, in order to be specific for *A2M-2* hnRNA the *A2M-2* antisense oligonucleotide is designed to target the pentanucleotide deletion found in *A2M-2*. In another embodiment of the invention, the *A2M-2* antisense oligonucleotide is designed to target *A2M-2* mRNA. The *A2M-2* deletion results in several variant mRNA transcripts with varying sequences. The *A2M-2* antisense oligonucleotides of the invention can be designed to target individual variants, or to target more than one of these variants. In addition, *A2M-2* antisense oligonucleotides targeting different *A2M-2* mRNA variants, or targeting *A2M-2* hnRNA, may be used either alone, or in conjunction with one another.

In addition, the *A2M-2* antisense oligonucleotide must be long enough so that it targets only *A2M-2*, but short enough to optimize delivery. Thus, the antisense oligonucleotide of the invention is preferably 8-50 nucleotides in length, and more preferably 15-30 nucleotides in length. Therefore, in one embodiment of the invention, the *A2M-2* antisense oligonucleotide is 8-50 nucleotides and is complementary to the coding strand of the region of *A2M-2* containing the site of the pentanucleotide deletion. In a preferred embodiment of the invention, the *A2M-2* antisense oligonucleotide is composed of 15-30 contiguous nucleotides of a region complementary to the site on the coding strand of *A2M-2* that contains the pentanucleotide deletion. In another embodiment of the invention, the *A2M-2* antisense oligonucleotide is composed of the last 8-50 contiguous nucleotides of SEQ ID NO:27. In a preferred embodiment of the invention, the *A2M-2* antisense oligonucleotide is composed of the last 15-30 contiguous nucleotides of SEQ ID

NO:27. In yet another preferred embodiment, the A2M-2 antisense oligonucleotide is composed of nucleotides 36-50 of SEQ ID NO:27. In another preferred embodiment of the invention, the A2M-2 antisense oligonucleotide is composed of nucleotides 20-50 of SEQ ID NO:27.

5 The A2M-2 antisense oligonucleotide may be DNA or RNA, *i.e.*, it may be composed of deoxyribonucleic acids or ribonucleic acids, respectively. Alternatively, the oligonucleotide may be composed of nucleotides with a phosphorothioate backbone to render the oligonucleotide more resistant to enzymatic degradation (van der Krol, A. R., *et al.*, *Biotechniques* 6:958-976
10 (1988); Cazenave, C. & Hélène, C., "Antisense Oligonucleotides," in: *Antisense nucleic acids and proteins: Fundamental and applications*, Mol, J. N. M. & van der Krol, A. R., eds., M. Dekker, publ., New York, pp. 1-6 (1991); Milligan, J. F., *et al.*, *J. Med. Chem.* 36:1923-1937 (1993)). In a preferred embodiment of the invention the A2M-2 antisense oligonucleotide is DNA.

15 Other modifications which may be used to protect the oligonucleotide include chemical changes to the 3' end of the oligonucleotide (van der Krol, A. R., *et al.*, *Biotechniques* 6:958-976 (1988); Khan, I. M. & Coulson, J. M., *Nucleic Acids Res.* 21:2957-2958 (1993); Tang, J. Y., *et al.* *Nucleic Acids Re.* 21:2729-2735 (1993)) or biotinylation of the 3' end followed by conjugation with avidin
20 (Boado, R. J. & Pardridge, W. M., *Bioconjugate Chem.* 3:519-523 (1992)). Alternatively, lipofection may be used to deliver the oligonucleotide, *i.e.*, packaging the oligonucleotide in lipid (McCarthy, M. M., *et al.*, *Endocrin.* 133:433-439 (1993b); Ogawa, S., *et al.*, *J. Neurosci.* 14:1766-1774 (1994)). Lipofection protects the oligonucleotide from nucleases and may aid in delivery
25 to the central nervous system.

 The A2M-2 antisense oligonucleotide can be easily synthesized by means of commercially-available automatic DNA synthesizers such as a DNA synthesizer manufactured by Applied Biosystems, or MilliGen, etc. In addition, methods of synthesizing oligonucleotides are well known in the art and are described, for
30 example, in *Oligonucleotides and Analogues a Practical Approach*, Eckstein, F., ed., Oxford University Press, publ. New York, (1991), and "Synthesis and

Purification of Oligonucleotides" in: *Current Protocols in Molecular Biology*, Ausubel, F. M., *et al.*, eds., John Wiley & Sons, Inc., publ., Vol. 1, §§ 2.11-2.12 (Suppl. 9 1993).

5 The invention also relates to pharmaceutical compositions containing the *A2M-2* antisense oligonucleotide, and one or more pharmaceutically acceptable carriers. In addition, the invention provides a method of treating AD and/or of suppressing *A2M-2* expression by administering the *A2M-2* antisense oligonucleotide to a subject. Preferably, the *A2M-2* antisense oligonucleotide is delivered to a subject who has been determined to be heterozygous or
10 homozygous for the *A2M-2* allele. Procedures for selecting and assessing subjects who are heterozygous or homozygous for *A2M-2* are described in Tanzi *et al.*, U.S. Serial No. 09/148,503, PCT Application No. PCT/US98/18535, and Blacker, D., *et al.*, *Nat. Genet.* 19:357-360 (August 1998). In another preferred embodiment of the invention, treatment of a subject with the *A2M-2* antisense oligonucleotide is done in conjunction with a therapy designed to replace or
15 supplement α_2 M function.

Antisense oligonucleotides have been safely administered to humans and several clinical trials are presently underway. Based on these clinical trials, oligonucleotides are understood to have toxicities within acceptable limits at
20 dosages required for therapeutic efficacy. One such antisense oligonucleotide, identified as ISIS 2105, is presently in clinical trials, and is used as a therapeutic against papillomavirus. Another antisense oligonucleotide, ISIS 2922, has been shown to have clinical efficacy against cytomegalovirus-associated retinitis *Antiviral Agents Bulletin* 5: 161-163 (1992); *BioWorld Today*, Dec. 20, 1993.
25 Therefore, it has been established that oligonucleotides are useful therapeutic agents and that they can be used for treatment of animals, especially humans.

The amount of the *A2M-2* antisense oligonucleotide administered to a subject will vary depending upon the age, weight, and condition of the subject. The course of treatment may last from several days to several months or until a
30 cure is effected or a diminution of disease state is achieved, or alternatively may continue for a period of years, for example, when used prophylactically. Optimal

dosing schedules can be calculated from measurements of drug accumulation in the body. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC₅₀'s in *in vitro* and *in vivo* animal studies. In general, dosage is from 0.01 mg to 100 g and may be given once daily, weekly, monthly or yearly.

Another therapeutic method of the invention is gene therapy to supplement α_2 M function. Because the *A2M-2* deletion may result in impaired α_2 M function, a strategy aimed at supplementing normal α_2 M, such as gene therapy, could serve as a means for treating, preventing or reversing AD. One embodiment of the invention is a viral vector carrying a transgene encoding wild type α_2 M, or an anti-LRP-A β peptide. Viral vectors suitable for use in the invention are those that are capable of transfecting nondividing, post-mitotic cells, and have low cytotoxicity. These vectors include, but are not limited to adenovirus, lentivirus, and HSV-1, but are preferably adeno-associated virus vector (AAV). AAV is a DNA virus that is not directly associated with any human disease, and therefore should present a lower risk of cytotoxicity (Freese, A. *et al.*, *Epilepsia* 38:759-766 (1997)). It can transfect nondividing, post-mitotic cells, such as neurons and dormant glial cells. In addition, there is some evidence that AAV may stably integrate into the host chromosome (Freese, Z. *et al.*, *Mov. Disord.* 11:469-488 (1996); Kaplitt, M. G. *et al.*, *Natur. Genet.* 8:148-154 (1994); Samulski, R. J., *et al.*, *J. Virol* 63:3822-3888 (1989); Kotin, R. M. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 87:2211-2215 (1990); Samulski, R. J. *et al.*, *E.M.B.O. J.* 10:3941-3950 (1991); Muzyczka, N., *Curr. Topics. Microbiol. Immunol.* 158: 97-129 (1992)). Recently, AAV was successfully used to deliver a reporter transgene to human hippocampal tissue (Freese, A. *et al.*, *Epilepsia* 38:759-766 (1997)).

Transgenes to be used in the viral vector include the full length cDNA encoding α_2 M (SEQ ID NO:1), or the anti-LRP-A β peptide described above. The construction of AAVlacZ is described by Kaplitt, *et al.*, and Samulski *et al.* (Kaplitt, M. G., *et al.*, *Nature Genet.* 8:148-154 (1994); Samulski, R. J., *et al.*, *J. Virol.* 63:3822-3888 (1989)). To insert the transgene into the viral vector, the

viral vector is first cut with restriction enzymes. PCR mediated integration is used to create corresponding restriction sites at the 3' and 5' ends of the transgene, and the transgene is ligated with AAV.

The invention also provides a method of combating AD by administering the viral vector carrying an α_2M , or an anti-LRP-A β peptide transgene and pharmaceutical compositions containing this viral vector.

The gene therapy of the invention can be administered using *in vivo* or *ex vivo* strategies. The *in vivo* approach involves the introduction of the viral vector directly into the tissue of the subject. *In vivo* methods of administration include direct injection into cerebrospinal fluid, or by stereotactic intracerebral inoculation into the hippocampus. In addition, some viral vectors, such as adenovirus, can be transported in a retrograde manner from the point of injection (Ridoux, V., *et al.*, *Brain Res.* 648:171-175 (1994); Kuo, H., *et al.*, *Brain Res.* 24:31-38 (1995)). Other routes of administration include nasal inhalation (Draghia, R., *Gene Ther.* 2:418-423 (1995)) and injection into the carotid artery after disruption of the blood brain barrier (Doran, S. E., *et al.*, *Neurosurgery* 36:965-970 (1995); Muldoon, L. L., *Am. J. Pathol.* 147:1840-1851 (1995)).

For the *ex vivo* approach, a suitable cell type, such as fibroblasts myoblasts, or neural progenitor cells, is harvested from a donor and grown in tissue culture. The cells are then transfected, and the cells harvested and implanted in the subject. *Ex vivo* methods are described, for example, at Raymon, H. K., *et al.*, *Exper. Neurol.* 144:82-91 (1997); Rosenberg, M. B., *et al.*, *Science* 244:1575-1578 (1988); Suhr, S. T., and Gage, F. H., *Arch. Neurol.* 50:1252-1268 (1993); Tuszynski, M. H., *et al.*, *Exp. Neurol.* 126:1-14 (1994); Ridoux, V. *et al.*, *Neuroreport* 5:801-804 (1994); Buc-Caron, M. H., *Neurobiol. Dis* 2:37-47 (1995); Sabaté, O., *et al.*, *Nat. Genet.* 9:256-260 (1995).

The amount of viral vector carrying a transgene administered to a subject will vary depending upon the age, weight, and condition of the subject. The course of treatment may last from several days to several months or until a cure is effected or a diminution of disease state is achieved, or alternatively may continue for a period of years, for example, when used prophylactically. Optimal

dosing schedules can be calculated from measurements of drug accumulation in the body. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. In general, dosage is from 1×10^4 to 1×10^{10} plaque forming units (pfu), but is preferably 1×10^6 to 5×10^7 pfu/kg and may be given once daily, weekly, monthly or yearly.

The therapeutic agents of the invention can be administered alone, or in concert with one another or with other therapeutic agents. For example, a subject may be treated with both the anti-LRP-A β molecule and the antisense oligonucleotide of the invention, to provide both a supplement of *A2M* function, and to block defective *A2M* function at the same time.

Suitable subjects for carrying out the present invention are typically male or female human subjects, and include both those which have previously been determined to be at risk of developing AD, and those who have been initially diagnosed with AD. The present invention may be employed in combating both familial AD (late onset and early onset) as well as sporadic AD. One preferable group of subjects are those who have been determined to be heterozygous or homozygous for the *A2M-2* allele. Procedures for selecting and assessing subjects who are heterozygous or homozygous for *A2M-2* are described in Tanzi *et al.*, U.S. Serial No. 09/148,503, PCT Application No. PCT/US98/18535, and Blacker, D., *et al.*, *Nat. Genet.* 19:357-360 (August 1998), all of which are herein incorporated by reference.

When the therapeutic agents as mentioned above are used as preventive or therapeutic agents for Alzheimer's disease, they may be made into preparations which satisfy the necessary requirements of the particular administering route together with usual carriers. For example, in the case of oral administration, preparations in the form of tablets, capsules, granules, diluted powder, liquid, etc. are prepared.

Pharmaceutical compositions containing the therapeutic agents of the invention, may be prepared in either solid or liquid form. To prepare the pharmaceutical compositions of this invention, one or more of the therapeutic agents is intimately admixed with a pharmaceutical carrier according to

conventional pharmaceutical compounding techniques, which carrier may take a wide variety of forms depending on the form of preparation desired for administration, for example, oral or parenteral. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material, or formulation auxiliary of any type. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed. Thus, for liquid oral preparations, such as for example, suspensions, elixirs and solutions, suitable carriers and additives include water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like; for solid oral preparations such as, for example, powders, capsules and tablets, suitable carriers and additives include starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like. In addition to such pharmaceutical carriers, cationic lipids may be included in the formulation to facilitate oligonucleotide uptake. One such composition shown to facilitate uptake is LIPOFECTIN (GIBCO-BRL, Bethesda, Md.).

Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are employed. If desired, tablets may be sugar coated or enteric coated by standard techniques. For parenterally injectable compositions, the carrier will usually comprise sterile, pyrogen-free water, or sterile, pyrogen-free physiological saline solution, though other ingredients, for example, for purposes such as aiding solubility or for preservatives, may be included. Parenterally injectable suspensions (for example, for intravenous or intrathecal injection) may also be prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed. See generally *Remington's Pharmaceutical Sciences* (18th ed.) Mack Publishing Co. (1990).

The pharmaceutical compositions of this invention may be administered in a number of ways depending upon whether local or systemic treatment is desired, and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral, for example, by intravenous drip, subcutaneous, intraperitoneal or intramuscular

injection or intrathecal or intraventricular administration. Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable. Compositions for intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

When necessary, the pharmaceutical composition may be prepared so that the therapeutic agent passes through the blood-brain barrier. One way to accomplish transport across the blood-brain barrier is to couple or conjugate the therapeutic agent to a secondary molecule (a "carrier"), which is either a peptide or a non-proteinaceous moiety. The carrier is selected such that it is able to penetrate the blood-brain barrier. Examples of suitable carriers are pyridinium, fatty acids, inositol, cholesterol, and glucose derivatives. Alternatively, the carrier can be a compound which enters the brain through a specific transport system in brain endothelial cells, such as transport systems for transferring insulin, or insulin-like growth factors I and II. This combination of therapeutic agent and carrier is called a prodrug. Upon entering the central nervous system, the prodrug may remain intact or the chemical linkage between the carrier and therapeutic agent may be hydrolyzed, thereby separating the carrier from the therapeutic agent. See generally U.S. Pat. No. 5,017,566 to Bodor.

An alternative method for transporting the therapeutic agent across the blood-brain barrier is to encapsulate the carrier in a lipid vesicle such as a microcrystal or liposome. Such lipid vesicles may be single or multi-layered, and encapsulate the therapeutic agent either in the center thereof or between the layers thereof. Such preparations are well known. For example, PCT Application WO 91/04014 of Collins et al. describes a liposome delivery system in which the therapeutic agent is encapsulated within the liposome, and the outside layer of the

liposome has added to it molecules that normally are transported across the blood-brain barrier. Such liposomes can target endogenous brain transport systems that transport specific ligands across the blood-brain barrier, including but not limited to, transferring insulin, and insulin-like growth factors I and II. Alternatively, antibodies to brain endothelial cell receptors for such ligands can be added to the outer liposome layer. U.S. Pat. No. 4,704,355 to Bernstein describes methods for coupling antibodies to liposomes.

Another method of formulating the therapeutic agent to pass through the blood-brain barrier is to prepare a pharmaceutical composition as described above, wherein the therapeutic agent is encapsulated in cyclodextrin. Any suitable cyclodextrin which passes through the blood-brain barrier may be employed, including β -cyclodextrin, γ -cyclodextrin, and derivatives thereof. See generally U.S. Pat. No. 5,017,566 to Bodor; U.S. Pat. No. 5,002,935 to Bodor; U.S. Pat. No. 4,983,586 to Bodor.

Another method of passing the therapeutic agent through the blood-brain barrier is to prepare and administer a pharmaceutical composition as described above, with the composition further including a glycerol derivative as described in U.S. Pat. No. 5,153,179 to Eibl.

An alternative method of delivering the therapeutic agent to the brain is to implant a polymeric device containing the agent, which device is able to provide controlled release delivery of the agent to the brain for an extended period after implantation. Examples of such implantable polymeric devices are described in U.S. Pat. No. 5,601,835 to Sabel, and in U.S. Pat. No. 5,846,565, to Brem.

Another aspect of the invention relates to methods of screening for therapeutic agents for AD that can replace or supplement normal α_2 M function and activities, and/or suppress defective α_2 M function.

The invention provides for a method of screening for therapeutic agents for AD that can suppress the production of RNA encoding α_2 M-2 variants, and thereby suppress the production of α_2 M-2 variants. One embodiment of the invention is a method for screening for therapeutic agents by incubating cells that are heterozygous or homozygous for *A2M-2*, and that express *A2M-2*, with a test

agent, and determining whether the agent increases the ratio of normal to aberrant *A2M* mRNA. Preferably the cells used are heterozygous for the *A2M-2* allele, with the other allele being *A2M-1* (*A2M-1/2* cells). Examples of cells that may be used for this assay include, but are not limited to, glioma cells, hepatocytes, and hepatoma cell lines. In addition, cells used for the assay may be transformed or transfected to enable them to grow indefinitely in culture. To screen for these agents, the cells carrying are incubated with the test agent, preferably, for a period ranging from 2 hours to 24 hours. The incubation period may be longer or shorter depending on the agent, and suitable incubation periods can be determined by one of ordinary skill in the art. Cells homozygous for *A2M-1* are used as a control. Procedures for *A2M-2* genotyping are described in Tanzi *et al.*, U.S. Serial No. 09/148,503, PCT Application No. PCT/US98/18535, and Blacker, D., *et al.*, *Nat. Genet.* 19:357-360 (August 1998). After incubation, the ratio of normal to aberrant α_2 M mRNA transcripts is determined, and compared to the ratio for cells (with the same genotype as the cells treated with agent) untreated with agent, and for *A2M-1/1* cells untreated with agent. An increase in the ratio of normal to aberrant α_2 M mRNA transcripts relative to this ratio for cells untreated with the agent would indicate an effective agent. This ratio for *A2M-1/2* cells untreated with an agent is typically from 5:1 to 20:1. If the ratio of normal to aberrant α_2 M mRNA transcripts approaches the ratio found in *A2M-1/1* cells untreated with agent, the agent will be considered effective. Thus, for example, if the ratio in *A2M-1/2* cells is 10:1, and the ratio in *A2M-1/1* cells is 100:1, a test agent that results in the ratio to 20:1 would be considered effective.

The ratio of normal to aberrant transcripts may be quantitated by S1 nuclease analysis, or by RT PCR on RNA isolated from the glioma cells. Protocols for RNA isolation for cells in culture, and for S1 nuclease analysis is described in "Preparation and Analysis of RNA" in: *Current Protocols in Molecular Biology*, Ausubel, F. M., *et al.*, eds., John Wiley & Sons, Inc., publ., Vol. 1, § 4 (Suppl. 37 1997). S1 nuclease analysis is performed using a single-stranded antisense probe encompassing at least exons 17-18 (bp 2057-2284 of SEQ ID NO:1), synthesized from a full length *A2M* cDNA template (SEQ ID

NO:1). Preferably, the probe would encompass exons 17, 18 and part of exon 19. The length of the probe is preferably from 250 bp to 500 bp long, and is more preferably 300 bp long. The probe may be up to 4353 bp (the length of the coding region), however, increasing the length of the probe may decrease the accuracy of the assay. In a preferred embodiment of the invention, the probe is complementary to nucleotides 2024-2323 of SEQ ID NO:1, in another preferred embodiment, the probe is complementary to nucleotides 2057-2384 of SEQ ID NO:1. After the RNA has been hybridized with the probe, and digested with S1 nuclease, samples are run on a polyacrylamide gel with molecular weight markers. Wild type mRNA transcript (*A2M-1*) should appear as a band corresponding to the length of the probe, for example, 300 bp, *A2M-2* variant transcripts should appear as smaller bands. Total normal mRNA to total variant mRNA is compared and the ratio of normal to aberrant determined.

Alternatively, RT PCR may be used to quantitate mRNA transcripts. Protocols for RT PCR are described in "The Polymerase Chain Reaction" in: *Current Protocols in Molecular Biology*, Ausubel, F. M., *et al.*, eds., John Wiley & Sons, Inc., publ., Vol. 2, § 15.4 (Suppl. 17 1992). RNA isolated from the treated and control cells is amplified using RT PCR with labeled primers designed to amplify a region including at least exons 17-18 (bp 2057-2284 of SEQ ID NO:1), and preferably exons 17, 18 and part of exon 19. In addition, the primers may be designed to target mRNA by synthesizing them so that they bind to the junction of two exons. For example, in a preferred pair of primers, the first primer would hybridize to *A2M* cDNA encoding the end of exon 16 and beginning of exon 17, and the second primer would hybridize to *A2M* cDNA encoding the end of exon 18, and beginning of exon 19. The primers may be from 8-50 nucleotides in length, but are preferably 15-30 nucleotides in length, and are more preferably 15 nucleotides in length. The PCR product is then run on a polyacrylamide gel with molecular weight markers. Bands corresponding to wild type mRNA transcripts should correspond to the length of *A2M-1* cDNA corresponding to the far ends of the primers used. For example, wild type mRNA amplified by primers designed to amplify the last 5 base pairs of exon 16 to the first 5 base pairs of exon

19 (bp 2052-2289 of SEQ ID NO:1), would be 238 nucleotides. If the primers were designed to amplify a region starting at the beginning of exon 17, including exon 18, and ending after the first 100 nucleotides of exon 19 (bp 2057-2456 of SEQ ID NO:1) the expected fragment length would be 400 nucleotides for normal mRNA. Variant mRNA transcripts will be shorter. Total normal mRNA to total variant mRNA is compared and the ratio of normal to aberrant determined.

Other methods of RNA quantitation that may be used in the invention are well known in the art, and are described in, *for example, PCR Protocols, A Guide to Methods and Applications*, Innis, A., *et al.*, eds., Academic Press, Inc., San Diego, CA, pub., pp. 60-75 (1990).

Another embodiment of the invention is to screen for nontoxic agents that can activate α_2 M through mechanisms other than cleavage of the bait domain. For α_2 M tetramers having one or more α_2 M-2 monomers, protease activation of the bait domain may be impaired. Because activation is required to expose the LRP binding domain, impairment of activation of one or more monomers of a tetramer would result in a decreased ability to bind to LRP. Consequently, these tetramers would be inefficient at clearing A β through LRP mediated endocytosis. However, α_2 M may be activated through mechanisms other than protease cleavage of the bait domain. For example, agents other than proteases, such as methylamine, activate α_2 M through the thiolester site. These agents would be able to activate defective α_2 M monomers, exposing the LRP binding domain (and other domains) and potentially allowing for LRP mediated clearance of A β . In addition, these agents could be used to increase the amount of active wild type α_2 M tetramers, to compensate for defective α_2 M tetramers. Presently, effective nontoxic agents capable of activating α_2 M at sites other than the bait domain are unknown. The invention provides for a method of screening for such agents.

To screen for these agents, α_2 M is treated with a test agent, and then tested to determine whether it has undergone a conformational change, or for its ability to bind to LRP. The α_2 M used for the assay may be wild type α_2 M, α_2 M-2, or α_2 M mutants that are missing all, or a portion of the bait domain. However, preferably, wild type α_2 M is used. In addition, α_2 M used for the assay may be in

the form of dimers or tetramers, but is preferably in the form of tetramers. For treatment of α_2 M with the test agent, the α_2 M is preferably incubated with the test agent for 2-24 hours. However, the incubation period may be longer or shorter according to the agent, and suitable incubation periods can be determined by one of ordinary skill in the art. To determine whether treated α_2 M has undergone a conformational change, the α_2 M electrophoretic-mobility assay may be used. To determine the ability of treated α_2 M to bind to LRP, any method of measuring LRP binding may be used, however, preferred methods include enzyme-linked immunosorbent assays (ELISA), immunoblotting, LRP mediated endocytosis, and LRP mediated degradation.

The α_2 M electrophoretic mobility assay can also be used to determine whether treated α_2 M has been activated, by determining whether treated α_2 M has undergone the conformational change expected for activated α_2 M. The α_2 M electrophoretic-mobility assay consists of analyzing the electrophoretic mobility of α_2 M under non-denaturing conditions after incubation with the test agent, or as a control, a protease, or other reagent capable of converting α_2 M to the fast form (Barret, A. J., *et al.*, *Biochem. J.* 181: 401-418 (1979); Bowen, M. E., and Gettins, P. W., *J. Biol. Chem.* 273:1825-1831 (1998)). α_2 M can exist in two forms easily distinguishable by mobility in gel electrophoresis (Barret, A. J., *et al.*, *Biochem. J.* 181: 401-418 (1979)). The difference in mobility is due to the conformational change that α_2 M undergoes after activation with a protease or other agent, such as methylamine. This conformational change results in an increase in electrophoretic mobility on poly-acrylamide gels run under non-denaturing conditions (this form is referred to as the "fast form" of α_2 M) (Barret, A. J., *et al.*, *Biochem. J.* 181: 401-418 (1979); Bowen, M. E., and Gettins, P. W., *J. Biol. Chem.* 273:1825-1831 (1998)). This "slow to fast" conversion is used as the basis for an assay for this conformational change, and the two different α_2 M conformations are referred to as the slow and fast forms (Bowen, M. E., and Gettins, P. W., *J. Biol. Chem.* 273:1825-1831 (1998)). Conversion from the slow to fast form for α_2 M treated with a test agent would indicate that the agent had

activated α_2 M. Where this assay is used to determine the effectiveness of a test agent, the α_2 M treated with the agent would preferably be tetrameric.

The α_2 M electrophoretic mobility assay and methods of purifying α_2 M from serum are described by Barret *et al.* in Barret, A. J., *et al.*, *Biochem. J.* 181: 401-418 (1979), and by Bowen *et al.* in Bowen, M. E., *et al.*, *Arch. Biochem. Biophys.* 337:191-201 (1997), and in Bowen, M. E., and Gettins, P. W., *J. Biol. Chem.* 273:1825-1831 (1998). After incubation with the test agent, the α_2 M sample may be run on polyacrylamide gel under nondenaturing conditions, such as those described in Bowen, M. E., *et al.*, *Arch. Biochem. Biophys.* 337:191-201 (1997). The α_2 M sample may be detected by methods well known in the art such as by radiolabelling the protease used, or by Western Blot using anti- α_2 M antibodies. Activated and unactivated α_2 M may be used as controls for comparison of electrophoretic mobility with the sample being analyzed.

In one embodiment of the invention, ELISA is used to determine the ability of treated α_2 M to bind to LRP. ELISA protocols are described in "Immunology" in: *Current Protocols in Molecular Biology*, Ausubel, F. M., *et al.*, eds., John Wiley & Sons, Inc., publ., Vol. 2, § 11.2 (Suppl. 15 1991). In this assay, microtiter plate wells coated with an anti- α_2 M IgG that recognizes only activated α_2 M, such as the antibody described by Marynen *et al.*, (Marynen, P., *et al.*, *J. Immunol.* 127: 1782-1786 (1981)), are incubated with the treated α_2 M, or control molecule. The wells are then incubated with an enzyme-conjugated anti- α_2 M IgG and rinsed. Next, the wells are incubated with the substrate for the enzyme conjugate, rinsed, and the amount of α_2 M sample bound in the well is determined. Alternatively, microtiter plate wells are coated with anti-LRP IgG and rinsed. The wells are then incubated with LRP and rinsed. This LRP is preferably soluble LRP. Then the wells are incubated with α_2 M treated with the test agent, untreated α_2 M, or activated α_2 M, and rinsed. Next the wells are incubated with enzyme-conjugated anti- α_2 M IgG, rinsed again, and then incubated with the substrate for the enzyme that is conjugated to the anti- α_2 M IgG. The amount of α_2 M sample bound in the well is then determined. In another embodiment, wells coated with LRP are incubated with α_2 M treated with the test agent, untreated unactivated

α_2 M, or untreated activated α_2 M, and rinsed. The wells are then incubated with enzyme-conjugated anti- α_2 M IgG, rinsed, and then treated with the enzyme substrate, and the amount of α_2 M sample bound is determined. The anti- α_2 M IgG may be conjugated with, for example, horseradish peroxidase, urease or alkaline phosphatase, but is preferably labeled with a fluorescent label, such as 4-methylumbelliferyl phosphate (MUP). The appropriate substrate is added to the wells, the wells are washed, and then quantitated with a microtitre plate reader.

Alternatively, the ability of α_2 M treated with the test agent to bind to LRP may be determined by immunoblotting methods. Unlabeled soluble LRP is incubated separately with α_2 M treated with the test agent, untreated unactivated α_2 M, and untreated α_2 M activated by methylamine or trypsin. Samples are then electrophoresed on a 5% SDS-PAGE, under non-reducing conditions, transferred to polyvinyl difluoride nitrocellulose membrane, and probed with anti- α_2 M IgG and anti-LRP IgG. If the α_2 M treated with the test agent may be detected by both anti- α_2 M IgG and anti-LRP IgG it can be concluded that the treated α_2 M can bind A β . In another method of immunoblotting, an antibody specific for the LRP binding domain of α_2 M, such as that described by Marynen, *et al.*, (Marynen, P., *et al.*, *J. Immunol.* 127: 1782-1786 (1981)), is used as the anti- α_2 M IgG, and the samples are not incubated with LRP. Recognition of the treated α_2 M by this antibody indicates that α_2 M has been activated.

In addition, the ability of α_2 M treated with a test agent to bind to LRP can be determined by its ability to undergo LRP mediated endocytosis using cell culture experiments as described by Kounnas *et al.* (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995); Kounnas, M. Z., *et al.*, *J. Biol. Chem.* 270:9307-9312 (1995)). Cells expressing LRP, mouse embryo fibroblasts, are incubated for 18 hours with 125 I-A β (alternatively, A β may be labeled with 3 H or 14 C) in the presence or absence of with α_2 M treated with the test agent, untreated unactivated α_2 M, and untreated α_2 M activated by methylamine or trypsin, in the presence or absence of RAP (400 nM). RAP is an inhibitor of LRP ligand binding, and is added to determine if endocytosis is LRP mediated. In addition, chloroquine (0.1 mM) is added to inhibit lysosomal degradation of 125 I-A β .

5 The amount of radioactive ligand released by treatment with trypsin-
EDTA, proteinase K solution defines the surface-bound material, and the amount
of radioactivity associated with the cell pellet defines the amount of internalized
ligand. Activated $\alpha_2\text{M}/^{125}\text{I-A}\beta$ will serve as positive control. Under the conditions
described, more than 4-8 fmoles/ 10^4 cells of activated $\alpha_2\text{M}/^{125}\text{I-A}\beta$ should be
10 internalized after 18 hours of incubation (Kounnas, M. Z., *et al.*, *Cell* 82:331-340
(1995)). Unactivated $\alpha_2\text{M}/^{125}\text{I-A}\beta$ and activated $\alpha_2\text{M}/^{125}\text{I-A}\beta$ in the presence of
RAP should not be internalized, therefore, no more than 2-4 fmoles/ 10^4 cells
should be internalized. If the amount of test agent treated $\alpha_2\text{M}/^{125}\text{I-A}\beta$ is greater
than 4-8 fmoles/ 10^4 cells, it can be concluded that $\alpha_2\text{M}/^{125}\text{I-A}\beta$ has the ability to
undergo LRP mediated endocytosis. In addition, unactivated $\alpha_2\text{M}/^{125}\text{I-A}\beta$, and
activated $\alpha_2\text{M}/^{125}\text{I-A}\beta$ in the presence of RAP should not be internalized, therefore
no more than 2-4 fmoles/ 10^4 cells should be internalized (Kounnas, M. Z., *et al.*,
15 *Cell* 82:331-340 (1995)). Internalization of the treated $\alpha_2\text{M}/^{125}\text{I-A}\beta$ complex will
be deemed abolished if treated $\alpha_2\text{M}/^{125}\text{I-A}\beta$, in the presence and absence of RAP,
and unactivated $\alpha_2\text{M}/^{125}\text{I-A}\beta$ show the same amount of radioactivity associated
with the cell pellet.

20 To determine the ability of treated $\alpha_2\text{M/A}\beta$ complexes to undergo
degradation after endocytosis, this cell culture protocol is repeated without
chloroquine. The radioactivity appearing in the cell culture medium that is soluble
in 10% trichloroacetic acid is taken to represent degraded $^{125}\text{I-A}\beta$ (Kounnas, M.
Z., *et al.*, *Cell* 82:331-340 (1995); Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911
(1997)). Total ligand degradation is corrected for the amount of degradation that
occurs in control wells lacking cells. Because free $^{125}\text{I-A}\beta$ can be degraded in an
LRP independent manner, degradation is measured for treated $\alpha_2\text{M}$, and untreated
25 $\alpha_2\text{M}$ complexes with $^{125}\text{I-A}\beta$, as well as for free $^{125}\text{I-A}\beta$, in the presence and
absence of RAP. Using the same positive and negative controls as above, if RAP
does not decrease the amount of TCA soluble radioactivity by at least 30% for the
treated $\alpha_2\text{M}/^{125}\text{I-A}\beta$ complex, it can be concluded that $^{125}\text{I-A}\beta$ ligand of treated
30 $\alpha_2\text{M}$ is not degraded.

It will be readily apparent to those skilled in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

Example 1

In view of the link between the inheritance of *A2M-2*, and the role of α_2 M in brain, the potential effects of the *A2M-2* deletion polymorphism on *A2M* mRNA and on the α_2 M protein were investigated. These studies were complicated by the fact the polymorphism does not directly alter the coding sequence of α_2 M, but consists of an intronic deletion just before the exon 18 splice acceptor site (Matthijs, G., *et al.*, *Nucleic Acids Res.* 19:5102 (1991)). If exon 18 were to be deleted as a result of the *A2M-2* polymorphism, this deletion would result in the loss of half of the active center or "bait" region of α_2 M (specifically, deletion of the last 20 amino acids out of the 39 amino acids forming the bait region), with likely adverse functional consequences for α_2 M activity. With specific regard to A β , the peptide does not directly bind to the bait region. However, recognition and cleavage of the bait domain by target proteases is a necessary prerequisite *in vivo* for activation of α_2 M via a conformational change in the α_2 M tetramer. Activation of α_2 M then results in the presentation of the LRP-binding domains which is essential for binding to LRP (Borth, W., *FASEB J.* 6:3345-3353 (1992)). Thus, clearance of α_2 M ligands (for example, cytokines, growth factors, A β), would be hampered by deletion of the bait domain (exon 18).

A specific deletion of exon 18 due to the *A2M-2* deletion would also result in a frame-shift in the coding region in exon 19, resulting in the synthesis of a truncated α_2 M monomer. Therefore, one likely consequence of a modification of the bait region is the formation of a defective α_2 M tetramer (insertion of defective monomer) which could not be activated and undergo subsequent endocytosis via

LRP. Experiments with an exon 18 deleted α_2 M construct expressed in cells indicate that a truncated α_2 M protein at the bait region can still be secreted and form tetramers with itself. In addition, only human glioma cell lines positive for the *A2M-2* allele produced altered *A2M* message and corresponding truncated α_2 M monomers consistent with a deletion of exon 18 followed by termination of the amino acid sequence in exon 19.

Methods and Results

First, the effect of the *A2M-2* deletion on RNA splicing and on α_2 M complex formation and secretion were investigated. To study the biological effects of the *A2M-2* polymorphism in an endogenous system, 15 human glioblastoma cell lines expressing high levels of α_2 M were genotyped (Blacker, D., *et al.*, *Nature Genetics* 19:357-360 (1998)). While the highest levels of α_2 M would be expected from hepatoma cell lines, glioblastomas were chosen because of their CNS origin. Ten primary glioblastoma cell lines (all derived from different patients) were homozygotes for the *A2M-1* (no deletion) allele, while 3 cell lines were *A2M-1/2* heterozygotes for the deletion. Two cell lines did not qualify for either of these alleles and were excluded from further studies. At the molecular level, the *A2M-2* allele consists of a deletion of 5 bp (ACCAT) in the consensus polypyrimidine tract immediately prior to the consensus 3' AG at the splice acceptor site of exon 18 (Matthijs, G., *et al.*, *Nucleic Acids Res.* 19:5102 (1991)). Given the position of the polymorphism, aberrant *A2M* RNA splicing might be expected to lead to a deletion at exon 18 since the consensus polypyrimidine tract would be reduced by 3 pyrimidines (to a minimal consensus configuration for exon splicing). Deletion of exon 18 would, in turn, result in termination of the protein due to a stop codon in exon 19. Reverse transcription-PCR (RT-PCR) was employed in attempts to identify aberrant splice products in the vicinity of exon 18 of the *A2M* gene. An expected 399 bp fragment encompassing exons 17, 18, and 19 was amplified by RT-PCR of RNA isolated from the 13 human glioma cell lines. Agarose gel/ethidium bromide staining was not sensitive enough to reveal aberrant *A2M* transcripts in any of the cell lines containing the *A2M-2* allele.

However, using polyacrylamide gels, various ^{33}P -labeled PCR products ranging in size between 250-290 bp were detected. These products were found exclusively in the *A2M*-1/2 cell lines (Figure 1).

5 Next, these products were cloned into the vector pCR 2.1. Four different clones representing aberrant mRNA transcripts have been identified using this approach (Figure 2). Sequencing of these clones revealed aberrant splicing events around exon 18 leading to the production of variably sized RNAs in which exon 17 and/or 19 may also be shortened. Clone 1 has a 208 bp deletion (2126-2334) including exon 18 and, interestingly, also 42 and 50 bp of exons 17 and 19, respectively. The protein product resulting from such a deletion would still be in frame with 69 amino acids missing, including most of the bait region. Clones 2, 3, and 4 contain unidentified DNA fragments which continue within exon 19 to bp 2355, 2320, and 2297 respectively. The unknown sequences are most likely intronic sequences that are not accessible in DNA databases. Therefore, aberrant splicing events around exon 18 do not appear to simply result in the precise deletion of exon 18. Rather, they lead to the production of variably sized RNAs in which exons 17 and/or 19 may also be partly deleted.

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Next, experiments designed to detect mutant forms of $\alpha_2\text{M}$ protein containing large deletions or truncations were performed. Based on the low level of aberrant mRNA transcripts, the expected amount of mutant proteins could be below detection or not recognized by the antibody used, since the antibody was raised against the holoprotein. Finally, a truncated or grossly altered protein may be targeted by the quality control system in the ER for degradation prior to secretion. These concerns were addressed by producing an *A2M* cDNA construct in which a stop codon is inserted in the middle of exon 18 and transfecting this construct into chinese hamster ovary (CHO) cells, which do not produce $\alpha_2\text{M}$ endogenously. As seen in Figure 3, both media and extracts from the transfected cells contained truncated and the control full-length $\alpha_2\text{M}$ protein products. The gels shown were run under denaturing but non-reducing conditions. Under these conditions, monomers of the truncated protein and monomers and dimers of the full-length protein were detected in the cell lysate. In the media, however, almost

all of the truncated protein formed tetramers, and dimers were barely detectable. Wild-type full-length α_2 M was also present in the media mainly in the form of tetramers and dimers. Besides demonstrating that the antibody used is able to recognize the N-terminal half of α_2 M and that a truncated α_2 M protein can be synthesized and secreted by CHO cells, the results of this experiment (Figure 3) also provided preliminary data indicating that secreted α_2 M levels may dramatically decrease as a result of the truncation.

Next, the effects of the *A2M-2* polymorphism on secretion and tetramer formation of endogenous α_2 M were examined. For this purpose, endogenous secreted α_2 M was analyzed by Western blot analysis. Glioblastoma cells were cultured overnight in OptiMem (Gibco) serum-free media (as bovine serum contains high levels of α_2 M), and secreted α_2 M was immunoprecipitated with a polyclonal α_2 M antibody obtained from Sigma. When the immunoprecipitate was resolved by SDS PAGE, the expected 180 kD monomer was detected in all lines tested, however, smaller aberrant forms of α_2 M were detected only in the *A2M-2* positive cells. Figure 4 shows cell lysates from wild-type and *A2M-2* deletion-bearing cells. The data revealed protein bands consistent with truncated forms of α_2 M exclusively in the *A2M-2* deletion-containing cells. The media (data not shown) from *A2M-1* and *A2M-2* cells contained primarily full-length α_2 M monomers, but in the media from the *A2M-2* cells small amounts of truncated species could also be observed.

Discussion

A reduced steady-state level of secreted α_2 M, or the presence of defective tetramers due to dominant negative effects of *A2M-2*, could result in impaired α_2 M function. Partial or total deletion of the sequences coding for the bait region in exons 17 and 18 are likely to modify protease binding, activation, and internalization of potentially defective tetramers containing mutant monomer(s). Therefore, the generation of very low levels of mutant monomers may have an amplified effect as one mutant monomer may potentially inhibit the function of three wild-type monomers in the tetramer (dominant negative effect). Based on

these and the linkage between the *A2M-2* deletion and AD (Blacker, D., *et al.*,
Nat. Genet. 19:357-360 (1998)), a critical role for α_2 M is indicated in AD
neuropathogenesis. The data described herein show that the *A2M-2* deletion leads
to deleted/truncated forms of α_2 M RNA and protein that may have a dominant
negative effect on normal α_2 M.

Example 2

To test the *A2M-2* antisense oligonucleotides of the invention, and the
S1 nuclease assay, *A2M-2* antisense oligonucleotides having the nucleotide
sequences of nucleotides 35-50, and 20-50 of SEQ ID NO:27 are synthesized
using an automatic DNA synthesizer (MilliGen). The oligonucleotides
recovered from 20% acrylamide-urea gel, and purified by means of an ethanol
precipitating method, and the precipitate is dissolved in water at a
concentration of 1 μ mol. *A2M-2* sense oligonucleotides complementary to
each of the antisense nucleotides are used as a positive control. Each of the
antisense or sense oligonucleotides (1 μ mol) is added to 1 ml cell culture
medium. Each 1ml sample is then incubated with glioma cells heterozygous for
the *A2M-2* allele, or homozygous for wild type *A2M* (*A2M-1*) at 37° C for 24
hours. The cells are washed with phosphate buffered saline, and homogenized
in a denaturing solution containing 4 M guanidine thiocyanate. RNA is
extracted using phenol/chloroform extraction and ultracentrifugation. The
RNA pellet is then rinsed with 1ml 75% ethanol/25% 0.1 M sodium acetate,
and resuspended in 100 μ l water. RNA from each sample is then probed using
a 300 bp antisense DNA probe encompassing exons 17 and 18 (nucleotides
2057-2356 of the full length cDNA for α_2 M (SEQ ID NO:1)) end labeled with
³²P. The probe is hybridized with 15 μ g RNA from each sample. The RNA is
then precipitated, washed and resuspended with S1 hybridization solution. The
samples are then denatured for 10 minutes at 65°C, and hybridized overnight at
30°C. 300 U S1 nuclease buffer in 150 μ l S1 nuclease buffer with single-
stranded calf thymus DNA is then added to each sample and incubated for 60
minutes at 30°C. The reaction is stopped, the RNA precipitated, washed, and

resuspended, and the samples are run on a polyacrylamide gel with molecular weight markers. Wild type transcripts (*A2M-1*) should appear as 300 bp bands, *A2M-2* variant transcripts should appear as smaller bands. Without *A2M-2* antisense oligonucleotide treatment, this ratio is expected to be approximately 10:1 wild type to variant mRNA. The ratio of wild type to variant transcripts is determined and compared to the ratio found for *A2M* mRNA from *A2M-1/1* cells.

Example 3

To screen for therapeutic agents capable of activating α_2 M through a site other than the bait domain, unactivated tetrameric α_2 M (Sigma) (about 1mg/ml) is incubated with 5, 20, 50 or 100 μ g of test agent in Tris/HCl or sodium phosphate buffer at 37°C for 2 hours. Untreated unactivated α_2 M, and untreated α_2 M activated with methylamine or trypsin are used as controls.

Microtiter plates are incubated for 2h at 37° with 50 μ l of LRP (10 μ g)/well, and then rinsed with deionized water. The plates are then filled with blocking buffer and rinsed. 50 μ l of treated α_2 M, untreated unactivated α_2 M, or untreated α_2 M activated with methylamine or trypsin is added to each well and incubated for 2h at room temperature. After rinsing, 50 μ l anti- α_2 M IgG conjugated with MUP in blocking buffer is added to the wells and incubated for 2h at room temperature. After rinsing, MUP substrate is added to the wells, and incubated for 1 h at room temperature. The amount of α_2 M bound is quantitated with a spectrofluorometer with a 365-nm excitation filter and 450 μ m emission filter.

Example 4

Given the evidence that only a few key interactions are required for α_2 M binding to LRP and A β (as discussed above), a small peptide containing LRP and A β binding domains could promote A β binding, LRP mediated endocytosis, and finally A β degradation. Such a peptide could serve as a substitute for α_2 M-2 if it is not able to promote A β clearance and degradation.

Protein-protein interactions are usually mediated by a few key interactions (Wells, J. A., *Proc. Natl. Acad. Sci. U.S.A.* 93:1-6 (1996)). The A β clearance properties of α_2 M do not require all the domains of an intact 5804 residue α_2 M tetramer. A 250-residue fragment of the α_2 M monomer contains both the A β and LRP binding domains (Hughes, S. R., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 95:3275-3280 (1998)). An 11-residue peptide can bind A β *in vivo* and a 27 residue LRP binding consensus sequence exists (Soto, C., *et al.*, *Nat. Med.* 4:822-826 (1998); Nielsen, K. L., *et al.*, *J. Biol. Chem.* 271:12909-12912 (1996); Soto, C., *et al.*, *Biochem. Biophys. Res. Commun.* 226:672-680 (1996)). A peptide containing an A β and an LRP binding domain could bind A β and target it for LRP mediated endocytosis followed by lysosomal degradation. To achieve this goal, first, a peptide consisting of an 11-residue A β binding peptide and a 27 residue LRP binding domain is produced and tested for A β binding and clearance properties. If necessary, the binding properties of this anti-LRP-A β peptide can be reoptimized using *in vivo* evolution techniques (Buchholz, F., *et al.*, *Nat. Biotechnol.* 16:657-662 (1998)).

Methods

Figure 6 shows the sequence of one possible anti-LRP-A β peptide. Using standard solid phase synthesis methods this peptide is synthesized in quantities sufficient to carry out tests to determine function in A β clearance. (See "Preparation and Handling of Peptides," in: *Current Protocols in Protein Science*, Coligan, J. E., *et al.*, eds., John Wiley and Sons, Inc., pub., Vol. 2., Chapter 18 (Suppl. 14 1998)). DNA encoding the fusion peptide is then synthesized. The DNA coding for the 27 residue LRP binding peptide is obtained by PCR amplification of codons 1366 to 1392 of the *A2M* gene (Nielsen, K. L., *et al.*, *J. Biol. Chem.* 271:12909-12912 (1996)). To integrate the 11 residue A β binding sequence into the LRP binding sequence PCR mediated insertion is used. A 55 nucleotide 5' PCR primer is designed that has 25 nucleotides of homology to the LRP binding sequence and 36 nucleotides

corresponding to the 11 residues of the A β binding peptide and a start codon. PCR mediated insertion is also used to insert an Xho I and Kpn I restriction enzyme sites at the 5' and 3' ends of the fusion gene, respectively. Cleavage with these enzymes will facilitate cloning of the fusion protein gene into (i) the pBAD/His expression vector (Invitrogen), for arabinose dependent expression of anti-LRP-A β in *E. coli*, and (ii) the pLex9-3H vector for use in the yeast three hybrid system (Tirode, F., *et al.*, *J. Biol. Chem.* 272:22995-22999 (1997)). The protein product, named anti-LRP-A β , of the resulting gene should have both A β and LRP binding properties.

A β Binding. The ability of anti-LRP-A β to bind A β is first determined by gel-filtration chromatography and immunoblotting. Both of these methods have been used successfully by other investigators to investigate A β binding to wild type and variant α_2 M (Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911 (1997); Du, Y., *et al.*, *J. Neurochem.* 69:299-305 (1997)). A β 1-42 is iodinated with 125 I, following the procedure of Narita *et al.* (Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911 (1997)). 125 I-A β (5 nmol) is incubated separately with anti-LRP-A β , unactivated α_2 M, unactivated α_2 M-2, α_2 M activated by methylamine or trypsin, or α_2 M-2 activated by methylamine or trypsin. A ten fold molar excess of A β is used and the samples are incubated in 25 mM Tris-HCl, 150 mM NaCl, pH 7.4 for two hours at 37°C. Controls containing only 125 I-A β are also incubated. The anti-LRP-A β / 125 I-A β , α_2 M/ 125 I-A β , and α_2 M-2/ 125 I-A β complexes are separated from unbound 125 I-A β using a Superose 6 gel-filtration column (0.7 X 20 cm) under the control of an FPLC (Pharmacia). 25 MM Tris-HCl, 150 mM NaCl, pH 7.4 are used to equilibrate the column and elute the samples. Using a flow rate of 0.05 ml/minute, 200 μ L fractions are collected. Having standardized the column with molecular weight markers ranging from 1000 kD to 4 kD, anti-LRP-A β / 125 I-A β , α_2 M/ 125 I-A β , and α_2 M-2/ 125 I-A β fractions are counted in a γ counter to determine the elution profile of 125 I-A β . If anti-LRP-A β has bound 125 I-A β , 125 I-A β should be detected by gamma counter at two peaks, one corresponding to the molecular weight of the

anti-LRP-A β /¹²⁵I-A β complex (about 8-9 kD for this anti-LRP-A β peptide), and one corresponding to the molecular weight of ¹²⁵I-A β (4.5 kD).

It is unlikely, but possible, that iodinated A β may lead to a false positive or negative binding. Therefore, immunoblotting experiments are undertaken to confirm the results of the gel-filtration chromatography experiment (Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911 (1997); Du, Y., *et al.*, *J. Neurochem.* 69:299-305 (1997)). Unlabeled A β is incubated separately with anti-LRP-A β , unactivated α_2 M, unactivated α_2 M-2, α_2 M activated by methylamine or trypsin, or α_2 M-2 activated by methylamine or trypsin, under the same conditions described above. Samples are electrophoresed on a 5% SDS-PAGE, under non-reducing conditions, and transferred to polyvinyl difluoride nitrocellulose membrane (Immobilon-P). These membranes are probed with polyclonal anti- α_2 M IgG or monoclonal anti-A β IgG. Immunoreactive proteins are visualized using ECL and peroxidase conjugated anti-rabbit IgG. Molecular mass markers are used to determine if the immunoreactive proteins from the anti- α_2 M and anti-A β blots for corresponding lanes display the same mobility. If the immunoreactive proteins display the same mobility then it will be concluded that A β binds anti-LRP-A β .

Endocytosis. The ability of anti-LRP-A β /A β complexes to undergo LRP mediated endocytosis and subsequent degradation is determined in cell culture experiments. The amount of radioligand that is internalized or degraded by cells has been described previously (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995); Kounnas, M. Z., *et al.*, *J. Biol. Chem.* 270:9307-9312 (1995)). Mouse embryo fibroblasts, which are cells that express LRP, are plated in 12 well plates to a density of 2×10^5 cells per well, and grown for 18 hours at 37°C in 5% CO₂. Cells are incubated in 1% Nutridoma (Boehringer Mannheim), penicillin/streptomycin, 1.5% bovine serum albumin for one hour prior to addition of ¹²⁵I-A β in the presence or absence of anti-LRP-A β , unactivated α_2 M, unactivated α_2 M-2, α_2 M activated by methylamine or trypsin, or α_2 M-2 activated by methylamine or trypsin, in the presence or absence of RAP (400 nM). To assess anti-LRP-A β /¹²⁵I-A β endocytosis by LRP,

chloroquine (0.1 mM) is added at the same time as anti-LRP-A β /¹²⁵I-A β (4 nM) to inhibit lysosomal degradation of ¹²⁵I-A β (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995)).

Following 18 hours of incubation with the anti-LRP-A β /¹²⁵I-A β , cells are washed with phosphate-buffered saline and treated with a trypsin-EDTA, proteinase K solution. Surface-bound material is defined as the amount of radioactive ligand released by this treatment, and the amount of internalized ligand is defined as the amount of radioactivity which remains associated with the cell pellet following the treatment.

Activated α_2 M/¹²⁵I-A β will serve as positive control. Under the conditions described, more than 4-8 fmoles / 10⁴ cells of activated α_2 M/¹²⁵I-A β should be internalized after 18 hours of incubation (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995)). Unactivated α_2 M/¹²⁵I-A β will serve as the negative control, because α_2 M must be activated by trypsin or methylamine to be recognized by LRP. If the amount of anti-LRP-A β /¹²⁵I-A β is greater than 2-4 fmoles/10⁴ cells, it can be concluded that anti-LRP-A β /¹²⁵I-A β has the ability to undergo LRP mediated endocytosis. Unactivated α_2 M/¹²⁵I-A β , and activated α_2 M/¹²⁵I-A β in the presence of RAP should not be internalized, therefore no more than 2-4 fmoles/10⁴ cells should be internalized (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995)). Internalization of the anti-LRP-A β /¹²⁵I-A β complex will be deemed abolished if anti-LRP-A β /¹²⁵I-A β , in the presence and absence of RAP, and unactivated α_2 M/¹²⁵I-A β show the same amount of radioactivity associated with the cell pellet.

Degradation. The experiment above to test endocytosis is repeated without chloroquine. The radioactivity appearing in the cell culture medium that is soluble in 10% trichloroacetic acid is taken to represent degraded ¹²⁵I-A β (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995); Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911 (1997)). Total ligand degradation is corrected for the amount of degradation that occurs in control wells lacking cells. Because free ¹²⁵I-A β can be degraded in an LRP independent manner, degradation is measured for anti-LRP-A β and α_2 M complexes with ¹²⁵I-A β as well as for free

^{125}I -A β in the presence and absence of RAP. Using the same positive and negative controls as above, if RAP does not decrease the amount of TCA soluble radioactivity by at least 30% for the anti-LRP-A β / ^{125}I -A β complex it can be concluded that ^{125}I -A β ligand of anti-LRP-A β is not degraded.

5 The anti-LRP-A β peptide may not promote A β binding and degradation because of steric constraints. If the anti-LRP-A β polypeptide does not promote A β binding and degradation another peptide is synthesized with a penta-glycine linker between the A β and LRP binding regions to provide the flexibility needed to bind both targets simultaneously. This anti-LRP-A β with linker is tested for A β binding, and LRP mediated endocytosis and degradation as described above. If this anti-LRP-A β does not provide for A β and LRP binding, the three hybrid system is used to reoptimize binding, and to screen for anti-LRP-A β with the ability to bind both A β and LRP.

10 The use of peptides in therapy is associated with two problems, transport across the blood-brain barrier, and the generation of an immune response. These problems can be minimized by shortening the peptide length. Thus when optimizing the anti-LRP-A β peptide, shorter binding domains may be preferred over longer domains, where binding capabilities are equally effective.

15 ***Yeast three hybrid system.*** The yeast three hybrid system is a genetic method to detect ternary protein complex formation (Figure 7) (Tirode, F., *et al.*, *J. Biol. Chem.* 272:22995-22999 (1997); Osborne, M.A., *et al.*, *Biotechnology* 13:1474-1478 (1995); Zhang, J. and Lautar, S., *Anal. Biochem.* 242:68-72 (1996); Licitra, E. J. and Liu, J. O., *Proc. Natl. Acad. Sci. U.S.A.* 93:12817-12821 (1996)). In the system, yeast growth only occurs when the "bait" recognizes both the "hook" and the "fish" (Figure 7). In this instance, the "hook" is constructed of the DNA coding for A β (Bales, K. R., *et al.*, *Nat. Genet.* 17:264 (1997)), fused to the coding sequence of the LexA DNA binding protein in pLex9-3H, a *TRP1* episomal vector (Tirode, F., *et al.*, *J. Biol. Chem.* 272:22995-22999 (1997)). The "fish" is constructed of the coding sequence for the 515kD extracellular domain of LRP, fused to the B42 activation domain

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in pVP 16, a *LEU2* episomal vector (Tirode, F., *et al.*, *J. Biol. Chem.* 272:22995-22999 (1997)). The "bait" is the DNA coding for anti-LRP-A β in the pLex9-3H vector, expression of anti-LRP-A β is repressed by methionine. These vectors are transformed into the L40 yeast strain. Transcription of the Leu 2 reporter gene occurs only when the A β fused DNA binding domain is brought into proximity to the transcriptional activation domain fused to LRP.

The A β /LRP binding fusion peptide should promote reporter gene transcription. The interaction between anti-LRP-A β and A β and LRP (515 kD) will be considered positive only if reporter gene expression (yeast growth) occurs when A β -LexA, LRP(515kD)-B42, and anti-LRP-A β are expressed. It is not likely that expression of A β -LexA will cause activation of the reporter transcription since this construct has been used successfully in the past. It is also unlikely that LRP(515kD)-B42 expression alone will cause reporter transcription, LRP(515kD) is not known to bind DNA. The interaction of A β -LexA and LRP(515kD)-B42 would cause reporter transcription and the A β parent protein APP is known to interact with LRP. However, the interaction between LRP and APP occurs via the Kunitz protease inhibitory domain far removed from the location of A β in APP (Kounnas, M. Z., *et al.*, *Cell* 82:331-340 (1995)). In addition biochemical evidence suggests that LRP does not recognize A β (Narita, M., *et al.*, *J. Neurochem.* 69:1904-1911 (1997)). Transformation of the A β -LexA and LRP(515kD)-B42 containing plasmids into EGY48 and monitoring the growth on media lacking leucine is carried out to insure that A β -LexA and LRP(515kD)-B42 do not interact. As positive controls the DNA sequence encoding the entire α_2 M monomer and the sequence encoding residues 1202-1451 of α_2 M are cloned separately into pLex9-3H, in place of anti-LRP-A β . The C-terminal fragment of α_2 M contains the full length A β and LRP binding domains (residues 1202-1451 of α_2 M) and it, along with the monomer, should give rise to reporter gene transcription.

If expression of anti-LRP-A β , A β -LexA, and LRP(515kD)-B42 does not activate reporter transcription then each of the binary interactions of anti-LRP-A β are tested in a traditional two hybrid screen. That is, concomitant

expression of anti-LRP-A β -B42 and A β -LexA, as well as anti-LRP-A β -B-42 and LRP(515kD)-LexA, is used to assess the ability of anti-LRP-A β to interact with A β -LexA and LRP(515kD)-LexA individually. If anti-LRP-A β interacts individually with both targets then one or all of the following is carried out: (i) a 5 residue glycine linker is added between the A β binding domain and the LRP binding to allow flexibility between the two binding domains, (ii) the A β -LexA and LRP(515kD)-B42 fusion partners are switched to become LRP(515kD)-LexA and A β -B42, and (iii) the polarity of the anti-LRP-A β is switched so that the LRP binding domain is N-terminal to the A β binding domain. If anti-LRP-A β interacts with one or neither of the targets, binding is reoptimized using random mutagenesis and selection by three hybrid screen for binding to both targets. The non-binding region of anti-LRP-A β is subjected to protein evolution techniques, error prone PCR and DNA shuffling (Buchholz, F., *et al.*, *Nat. Biotechnol.* 16:657-662 (1998)), followed by selection of constructs that bind target proteins. This is repeated until target binding is achieved.

Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in medicine, genetics, molecular biology, biochemistry, pharmacology and/or related fields are intended to be within the scope of the following claims.

All publications and patents mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patents mentioned are herein incorporated by reference to the same extent as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications can be practiced within the scope of the appended claims.